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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*
The purpose of this project was to determine whether chronic activation of brain corticotropin-releasing factor (CRF) stress systems led to oxidative damage of brain dopamine systems and to investigate individual susceptibility to this pathological cascade. In Specific Aim 1, the goal was to explore the effects of chronic psychological and physical stress on neuropharmacological, neurochemical and oxidative stress measures of mesocortical and nigrostriatal dopaminergic system integrity. Chronic psychological stress was found to produce activation and temporary dysfunction of the mesocortical and nigrostriatal dopaminergic systems, changes that were accompanied by evidence of increased lipid peroxidation, a marker of oxidative stress. In Specific Aim 2, the goal was to selectively breed rats for high and low response to stressors on the basis of their HPA-axis response to footshock. Two replicate lines have been and continue to be developed. The lines have statistically diverged and show good-to-excellent reproducibility of their ACTH responses to stress. Preliminary results suggest that they also may differ in their behavioral responses to stress. These lines will be a major resource for comparing the pathophysiologic effects of chronic psychological stress in stress hyper-responsive and hypo-responsive populations.

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INTRODUCTION:

Enduring dysregulation of stress response systems afflicts as many as one-third of combatants in major conflicts, and disorders of the stress response systems related to military operations result in substantial personal and public costs. The purpose of the present proposal was to explore the effects of chronic stress on neuronal pathology utilizing behavioral, neurochemical, molecular and genetic levels of analyses. The specific hypothesis under test was that chronic stress produces lasting changes in brain dopamine function. These changes may lead to neuronal damage through oxidative stress mechanisms, with certain subject populations being uniquely susceptible to this pathological cascade due to hyperresponsiveness of their brain corticotropin-releasing factor (CRF) stress response systems. To test this hypothesis, the following two specific aims have been pursued in male rats: 1) To examine the effects of chronic stress on behavioral, neurochemical, and molecular measures of neuronal pathology to the brain dopamine system; and 2) To develop a phenotype of hyperresponsiveness of the hypothalamic-pituitary-adrenal (HPA) axis by selective breeding. As proof-of-concept, we observed that administration of ferrous citrate, an oxidizing agent, produced changes in neuropharmacological responses consistent with impaired striatal dopaminergic functioning. Likewise, chronic administration of CRF, a form of psychological stress, but not chronic ether stress, a physical stressor, produced transient, functional changes in responses to neuropharmacologic probes reminiscent of impaired striatal dopaminergic functioning. Both chronic administration of CRF and repeated, but not single, defeat produced neurochemical changes consistent with increased dopamine turnover / metabolism in striatum and/or prefrontal cortex. In addition, both of these forms of chronic psychological stress were associated with transient tissue depletion of dopamine in prefrontal cortex, but not striatum. Repeated, but not single, defeat significantly increased free maldonialdehyde (MDA) levels, a marker of recent lipid peroxidation that is a product of oxidative stress, in both striatum and prefrontal cortex. To a lesser degree, chronic administration of CRF also transiently increased total MDA levels in prefrontal cortex. Collectively, these findings support the hypothesis that chronic psychological stress activates the mesostriatal and mesocortical dopaminergic systems, increases oxidative stress in their projection fields, with somewhat more persistent functional consequences, as reflected in hypodopaminergic responses to neuropharmacological challenge. In addition, during the funding period, Replicate lines that differ in their ACTH responses to stress have been and continue to be developed. Lines have statistically diverged and show good-to-excellent intersession consistency in

ACTH responses, confirming the replicability of the phenomenon. Preliminary results suggest that the differential responsiveness of these sub-lines to stress is general. That is, they differ in their ACTH responses to other stressors as well as in their behavioral responses to stressful environments. Future studies can examine the effects of chronic psychological stress on neuronal pathology in dopaminergic systems in these differentially stress-responsive populations.

BODY:

Specific Aim 1: To measure the effects of chronic stress on behavioral, neurochemical and molecular changes in neuronal pathology to the brain dopamine systems.

Experiment 3: Effect of chronic CRF on animal models sensitive to nigrostriatal DA system damage.

Overview – As described below, twenty-eight rats were implanted with chronic intracerebroventricular cannulae and subjected to daily i.c.v. treatment with CRF or vehicle for 13 days. During the month following treatment, they were assessed in behavioral measures sensitive to perturbation of the nigrostriatal DA system.

Subjects

Male Wistar rats (n=28; Charles River, Kingston NY), weighing 200-225 g at the start of the experiment were used. Under halothane anesthesia, rats were implanted with chronic indwelling intracerebroventricular (ICV) guide cannulae according to established methods [1, 19]. The coordinates were: AP, + 0.6 mm from bregma; L, $\pm$ 2.0 mm from the midline; DV, -3.2 mm from the skull surface, with the incisor bar 5 mm above the inter-aural line [23]. Rats were allowed 7 days to recover from surgery before treatment.

Drugs and treatments

For ICV injections, five microliters of either saline or CRF (1μg/5μl solution) was infused over an approximately 60 s period by gravity. To prevent efflux, the injector was left in place for an additional

30 s before the stylet again was placed in the guide cannula. Rats were injected daily with CRF or vehicle for 13 days.

d-Amphetamine sulfate (Sigma Chemical Co.; St. Louis, MO) and eticlopride, a D-2 receptor antagonist (RBI; Natick, MA) were injected subcutaneously (SC in isotonic saline) at a volume of 1.0 ml/kg of body weight.

Behavioral procedure

d-Amphetamine-induced stereotyped behavior

Stereotyped behavior was monitored in familiar, wire-mesh test cages measuring 20x25x36 cm. For assessment of non-specific locomotor behavior, cages also were equipped with photocells along the long axis, 2 cm above the floor of the cage. Background noise was provided by a white noise generator. Rats (14 saline and 14 CRF-treated) were habituated to the test cages for three hours for two consecutive days prior to the experiment day to overcome the potentially stressful nature of a novel environment. At different time points -- 1 day, 1 week and 1 month after chronic ICV treatment -- the rats were placed in the test activity cages for 90 min and then injected SC with 4.0 mg/kg d-amphetamine. Stereotyped behavior was rated for 180 min after the injection. During the 3-h test each rat was observed every 10 min for about 10 s. Stereotyped behavior was rated according to the Creese and Iversen [5] rating scale. This scale rates the intensity of stereotypy on a 7-point scale. The scores are defined as follows:

0: asleep or stationary
1: active
2: predominantly active, bursts of stereotyped sniffing or rearing
3: stereotyped activity, sniffing along fixed path of cage
4: stereotyped sniffing or rearing maintained in one location
5: stereotyped behavior in one location with bursts of gnawing or licking
6: continual gnawing or licking of cage bars

Stereotyped behavior was rated by one observer naïve to the rats' experimental treatment. Non-specific locomotor behavior was determined automatically by an IBM PC-compatible microcomputer that monitored photocell interruptions.

Catalepsy test

Catalepsy was measured using the bar test according to Pulvirenti and Koob [24]. Rats were injected with the DA D-2 receptor antagonist, eticlopride (0.05 mg/kg SC). This dose of eticlopride was chosen based on the results of a pilot study in which four doses (0, 0.025, 0.050 and 0.1 mg/kg s.c.) of eticlopride were administered in a Latin square design. Four hours after injection both of the rat's forepaws were placed on a bar 9 cm from the floor. The time elapsed until the rats repositioned both forepaws on the floor was recorded by an experimenter blind to the treatment condition. A cut-off of 5 min per observation was used. Control rats typically step down within a few seconds, whereas eticlopride-treated rats remain with their paws on the bar significantly longer (i.e., a cataleptic-like effect). Preliminary experiments found that repeated injection of eticlopride induced sensitization to its cataleptic effects, as has been similarly observed by others [3]. Therefore, rats were tested only at one time point -- 2 days after chronic CRF or saline treatment. Preliminary experiments demonstrated that exposure to amphetamine 24 hours earlier did not interfere with the cataleptic effect of eticlopride.

Statistical analysis

Stereotypy ratings were analyzed using the information statistic [16, 25]. This statistical analysis is analogous to χ^2, but is not constrained by small-cell frequencies. Stereotyped behavior rating was analyzed in two ways. First at each of the 18 time intervals the number of animals that displayed a score of 5 or 6 of the Creese and Iversen index [5] was processed and these 18 2I values were added to give a total 2I. For the analysis of the mean of the percentage of rats displaying a score of 5 or 6 over the 3 hours, Student's t-test was used. Catalepsy was analyzed using the Mann Whitney-U test for non-parametric measure.

Results

Effect of chronic CRF treatment on stereotyped behavior induced by d-amphetamine

Chronic CRF treatment (1 μg/day ICV for 13 days) reduced the stereotyped behavior induced by d-amphetamine (4 mg/kg s.c.) for one day after the treatment. Figure 1 shows the percentage of rats displaying stereotyped behavior rated 5 or 6 over the 3-h test period at 1 day after the CRF chronic treatment. This measure reached a maximum level at 80-90 minutes after amphetamine injection and then decreased to a minimum three hours later. The overall information statistic was significant ($2I=45.31$, $df=1,18$ $p<0.01$). Similarly, the mean rating for stereotyped behavior over the 3-h test was

significantly lower in the CRF group compared to the control group ($p<0.001$, Student's t-test). The stereotyped response to d-amphetamine at 1 week post-CRF treatment did not differ significantly between groups, although there was a trend to lower values in the CRF-group (see Figure 2). Finally, no differences were present at 1 month after the treatment (see Figure 3). Tables 1, 2 and 3 show the median of the value of all scores for each time point. Locomotor activity was recorded during the 90 minutes of habituation and during the 3-hr testing. No differences were observed between groups at any time point (data not shown).

Effect of chronic CRF treatment on catalepsy induced by eticlopride

Chronic CRF treatment (1 μg/day ICV for 13 days) increased the cataleptic effect of 0.05 mg/kg eticlopride, a D2 receptor antagonist, 2 days after the treatment (see Figure 4) ($p<0.05$ CRF-treated rats vs. controls, Mann Whitney-U test).

Summary of Experiment 3

Chronic administration of CRF, the stress-related neuropeptide, produced behavioral evidence of hypofunction of the nigrostriatal dopamine system as revealed by neuropharmacological probes. Hypodopaminergic function was reflected in reduced sensitivity to the ability of d-amphetamine to induce stereotyped behavior and increased sensitivity to the cataleptic effects of eticlopride. Signs of hypodopaminergic function normalized with time, but were evident for at least two days following cessation of CRF treatment.

Proof-of-concept Experiment: Effects of ferrous-citrate ICV infusion on stereotyped behavior induced by amphetamine.

To validate that oxidative stress in the brain induced an impairment of the dopaminergic system, a group of rats were acutely ICV injected with ferrous-citrate, an oxidizing agent, and tested after 7 days and 1 month in the stereotypy amphetamine test as described in Experiment 3. A group of 12 rats (6 per group) were injected ICV with 10 nmol of ferrous-citrate in saline, while the control group received saline. Rats were tested 7 days after ICV iron infusion.

Ferrous-citrate treated rats showed a lower stereotypy response to amphetamine either 7 days or 1 month after the infusion (see Figure 5). No differences in the basal locomotor activity were present

between control and iron-treated rats (not shown). These behavioral data indicate that oxidative stress impairs the dopaminergic system, as reflected in a blunted stereotyped behavioral response to amphetamine, and support the notion that the dopaminergic system is especially vulnerable to oxidative insults.

Experiment 4: Effects of chronic stressors on neurochemical measures of DA dysfunction.

Overview – As described below, two separate experiments were performed under this sub-aim. In Experiment 4a, twenty-six rats were implanted with chronic intracerebroventricular cannulae and subjected to daily ICV treatment with CRF or vehicle for 13 days. Rats in this study were sacrificed at 1 day, 1 week or 1 month following the end of CRF treatment and selective dopamine projection areas (prefrontal cortex, striatum and nucleus accumbens) were assayed for levels of DA and DOPAC. In, Experiment 4b, rats were subjected to repeated (n=8) or a single social defeat (n=6), a potent, ecologically relevant form of psychological stress, and compared to controls (n=7). Tissue levels of DA and DOPAC were examined in the striatum and prefrontal cortex 1 day following completion of the stress protocol.

Subjects

Male Wistar rats (n=47; Charles River, Kingston NY), weighing 200-225 g at the start of the experiments, were used. Those in the chronic CRF study were implanted with chronic indwelling ICV cannulae as described for Experiment 3 and allowed 7 days to recover from surgery before treatment.

Experimental Protocols

Experiment 4a - Chronic CRF exposure

CRF or vehicle was administered daily as described in Experiment 3 for 13 days.

Experiment 4b – Repeated psychological stress

Rats were randomly assigned to one of three conditions, repeated defeat (n=8), single defeat (n=6), or controls (n=7). In the repeated defeat condition, rats were subjected to a social defeat every other day for 21 days (i.e., total of 11 defeats) using the resident-intruder paradigm wherein the animal to be stressed was the intruder. Rats in the single defeat condition were removed from and immediately

returned to their cages on each of these occasions, except for Day 21, on which they were subjected to a social defeat. Controls were removed from and immediately returned to their cages on each occasion.

Defeat consists of the "intruder" rat being placed in the home cage of a larger, territorial, veteran, Long Evans male rat ("resident"). Upon submission by the intruder, the intruder rat is placed within a wire-mesh cage that remains within the resident's enclosure for an additional 30 min. Residents typically continue to exhibit aggressive, dominating behaviors towards the intruder during this interval, but can no longer attack them. Under our testing conditions, residents gain defeats over intruders with very short latency (<1 min) and with minimal or no physical trauma to the intruder. As a result, the intruder's stress is primarily psychological, rather than physical, and may result from the experience of defeat and continued perception of threat.

Analysis of total tissue DA and DOPAC levels

Tissue was punched from 1 mm coronal brain slices obtained using a precision wire brain matrix. For the chronic CRF study, unilateral tissue samples were ultrasonicated and centrifuged in one ml of 0.1 N perchloric acid containing 50 nM methylserotonin as an internal standard. The contralateral region was used for analysis of malondialdehyde (MDA), a product of lipid peroxidation. For the chronic psychological stress study, bilateral tissue samples were processed using a recently validated protocol that allows simultaneous assessment of monoamines and MDA from a single specimen [11]. Briefly, samples were ultrasonicated in 300 µl 1.15% KCl supplemented with 0.4 mM sodium azide. Following centrifugation, 0.4 N perchloric acid is added to an aliquot of the supernatant on a 1:1 (v/v) basis. Supernatants from each study were analyzed for DA and DOPAC. Concentrations of DA and DOPAC were determined with a microbore HPLC system equipped with a Spherisorb C-18 column (100 x 1 mm, 3 µm spheres) using a mobile phase composed of a 54 mM dibasic sodium phosphate buffer with 12% methanol (v/v), 0.2 mM EDTA, 0.9 mM 1-decanesulfonic acid and 4.9 mM triethylamine, at a final apparent pH of 4.8, pumped at 25 µl/min by an ISCO model 500D syringe pump. The monoamines were detected using a glassy carbon electrode set at +700 mV vs. Ag/AgCl by an amperometric detector (Princeton Applied Research model 400). The detection limit for each monoamine was approximately 0.2 nM. Monoamine levels were normalized to tissue protein content, which was determined by a modification of the Lowry method.

Statistical Analysis

Data were analyzed using a one-way factor analysis of variance (ANOVA) using the appropriate comparison conditions as between-subject factors (CRF study: controls, 1 day, 1 week or 1 month post-CRF treatment; Stress study: repeated, single or non-stressed controls). Pairwise comparisons to interpret main treatment effects were performed using a Fisher's post-hoc test.

Results

Effect of chronic CRF on DA and DOPAC levels

As shown in Figure 6A, tissue DA levels in the prefrontal cortex were significantly lower at 1 day and 1 week after the chronic CRF treatment compared to control animals (overall one-way ANOVA: $F[3,21]=7.3$ $p<0.005$; Fisher's post-hoc test: $p<0.005$ 1 day and 1 week vs. control). DA levels returned to baseline one month after CRF treatment. In the nucleus accumbens (Figure 6B), DA levels were significantly elevated at 1 week after the treatment (overall one-way ANOVA: $F[3,22)]=4.6$ $p<0.05$; Fisher's post hoc test $p<0.005$: 1 week vs. control). No significant differences in DA levels were found in the striatum at any time point (Figure 6C).

The DOPAC/DA ratios were significantly increased at 1 week after the treatment both in PFC (overall one-way ANOVA: $F[3,21] =5.8$ $p<0.005$; Fisher's post hoc test: $p<0.005$ 1 week vs. control) and striatum (overall one-way ANOVA: $F[3,22]=3.7$, $p<0.05$; Fisher's post hoc test: $p<0.01$ 1 week vs. control) (Figure 6E and 6F). One-way ANOVA revealed an overall effect of CRF treatment in the nucleus accumbens on the DOPAC/DA ratio [$F[3,23]=3.12$, $p<0.05$]. The ratio here followed a temporal profile resembling that in the striatum, but no pairwise comparisons were significant in post hoc analysis (Figure 5D). In agreement with the behavioral data from Experiment 3, all changes were transient, as values returned to baseline levels within one month of cessation of CRF treatment.

Effect of repeated or single defeat on DA and DOPAC levels

As shown in Figure 7, tissue DA levels in the striatum of repeated or singly defeated rats did not differ significantly from those of controls 1 day following their final defeat experience ($F[2,18]=0.56$ $p>0.55$). However, as shown in Figure 7, repeated, but not single defeat, produced almost 3-fold increased striatal DOPAC content one day following the final defeat experience ($F[2,18]=6.31$ $p<0.01$;

Fisher's post hoc test: $p<0.01$ vs. both singly defeated and control rats). Both repeated and acute defeat led to the well-established depletion of DA and elevation of DOPAC in the PFC ($p's<0.01$ vs. controls, data not shown).

Summary of Experiment 4

Chronic CRF treatment transiently (at 1 day and 1 week) reduced DA levels in the prefrontal cortex, but not in the striatum or nucleus accumbens. Likewise, both repeated and single defeat reduced PFC, but not striatal, DA levels one day following the final defeat. Both chronic CRF treatment and repeated defeat increased markers of dopamine system metabolism, albeit with a possibly different time course. Increased dopamine system turnover was evident 1 week post-CRF in the striatum and prefrontal cortex. Repeated defeat also elevated PFC and striatal DA system turnover/metabolism. In striatum, for example, levels of DOPAC, but not DA, were increased almost 3-fold one day following cessation of the stress protocol. Because of the longer duration of the repeated defeat protocol (21 vs. 13 days), the apparently different onset of changes in DA metabolism (1 vs. 7 day following the last defeat and CRF treatment, respectively) may simply reflect the similar latency from the initial treatments (20-22 days), as opposed to a different time course in the response to the treatments.

Experiment 5: Effects of chronic stressors on measures of oxidative stress and pathology.

Overview – Brain regions from rats in Experiment 4 also were analyzed for malondialdehyde (MDA). Oxidative damage to lipids results in the production of lipid peroxides. Such molecules are unstable and decompose to form several reactive aldehydes, one of the most abundant of which is MDA. MDA is a highly reactive dialdehyde, produced by lipid peroxidation and prostaglandin biosynthesis. It is a well-validated marker of oxidative stress induced by many forms of neurotoxicity [8, 11].

Malondialdehyde assay

Unilateral regions from rats chronically treated with CRF were homogenized in 10% w/v in phosphate-buffered saline (PBS) containing butylated-hydroxytoluene (BHT) (5 mM). Bilateral samples from rats in the repeated defeat study were ultrasonicated as described in Experiment 4b using sodium azide as the antioxidant and also were supplemented with BHT (5 mM final concentration) following centrifugation. Malondialdehyde (MDA) levels were determined using a colorimetric assay

(Bioxytech MDA-586 from OXIS International, Portland, OR). With this method N-methyl-2-phenylndole is allowed to react with MDA at 45°C. One molecule of MDA reacts with 2 molecules of N-methyl-2-phenylndole to yield a stable chromophore with maximal absorbance at 586 nm. Total MDA (free+adducted) levels were determined in the chronic CRF study following a hydrolysis step. Only recently has the measurement of free MDA levels been possible, as previous methods relied on the measurement of MDA as a thiobarbituric acid derivative. These studies show that free MDA levels reflect more recent, as opposed to both recent and previous, oxidative stress [4] and are more pathophysiologically relevant as they reflect the portion of MDA most available to perturb lipid membranes [14]. Therefore, free MDA levels were examined in the repeated defeat study without the hydrolysis step. MDA concentrations were normalized to tissue protein content, as determined by a modified Lowry method.

Statistical Analysis

Data were analyzed with a one-way factor analysis of variance (ANOVA) using the appropriate comparison conditions as between-subject factors (CRF study: controls, 1 day, 1 week or 1 month post-CRF treatment; Stress study: repeated, single or non-stressed controls). Pairwise comparisons to interpret main treatment effects were performed using a Fisher's post-hoc test.

Results

Effect of chronic CRF on total malondialdehyde levels

Chronic CRF treatment significantly decreased total MDA levels in the PFC (Figure 8) at 1 week post-CRF treatment ($F[2,12]=5.4$ $p<0.01$; Fisher's post-hoc test $p<0.05$ 1 week vs. control). This decrease was preceded by a non-significant increase in total MDA levels one day following chronic CRF treatment (Figure 8). Chronic CRF treatment did not significantly affect total MDA levels in the nucleus accumbens or striatum at any time point.

Effect of repeated or single defeat on free malondialdehyde levels

As shown in Figure 9, repeated defeat significantly increased the levels of free MDA present in striatum ($F[2,18]=3.41$, $p=0.05$; Fisher's post hoc $p<0.05$ vs. controls) 1 day following the final defeat. Whereas only 2/7 (28%) controls had detectable levels of free MDA in striatum, 6 of 8 rats subjected to repeated defeat did (75%) ($\chi^2[1]=3.23$, $p=0.07$). In contrast to striatum, baseline levels of free MDA

were detectable in prefrontal cortex samples from all subjects. As in striatum, repeated defeat reliably increased free MDA levels in prefrontal cortex (see Figure 9B, p=0.01 vs. controls). Single defeat did not reliably increase free MDA levels in either region, although levels were marginally higher in these subjects as well.

Summary of Experiment 5

Chronic administration of CRF tended to produce initial increases in total MDA levels in prefrontal cortex, which were followed 6 days later by significantly reduced total MDA levels, possibly reflecting compensatory increases in antioxidant activity. Repeated, but not single defeat, was associated with reliably increased free MDA levels in both striatum and prefrontal cortex one day following the final defeat. The more robust signs of lipid peroxidation observed in the repeated stress study may reflect the greater sensitivity and specificity of the free MDA measure to recent oxidative stress or differences in the oxidative consequences of the experimental treatments.

Experiment 2: The effect of chronic physical stress on animal models sensitive to nigrostriatal system damage.

Male, Wistar rats were subjected daily to 3 minutes of ether stress (n=6) or identical handling without ether stress (n=8) for 10 days. Catalepsy-like responses to eticlopride and stereotyped behavior responses to d-amphetamine were assessed as in Experiment 3. As shown in Figure 10 and Table 4, the results showed no reliable difference between the groups at 1 day or 1 week post-stressor exposure. However, physically stressed subjects tended to show effects in the same direction as those observed in rats treated chronically with CRF.

Interpretation of Results – Specific Aim 1

As proof of concept, it was observed that central infusion of ferrous citrate, an oxidizing agent, produced behavioral signs of hypodopamingergic functioning, most specifically, an attenuated stereotyped response to d-amphetamine. This finding supported the underlying hypothesis that dopaminergic systems are especially vulnerable to oxidative insults that have functional consequences.

Additional studies examined the effects of the stress-related neuropeptide corticotropin-releasing factor (CRF) on the same substrates. Chronic CRF treatment (1μg/day for 13 days) led to transient behavioral alterations consistent with hypodopamingergic functioning, including an exaggerated cataleptic-like response to dopamine receptor antagonism and a blunted stereotyped response to amphetamine. Correspondingly, chronic CRF treatment decreased DA levels in the PFC, and increased DA metabolism/turnover was in striatum and PFC at 1 day and 1 week post-treatment. In agreement with the behavioral observations, monoamine measures normalized within 1 month of treatment. Chronic CRF treatment also marginally increased total MDA levels, an index of oxidative damage, in prefrontal cortex, with signs of compensatory recovery evident by 1 week post-treatment. These data show that chronic elevation of brain CRF levels leads to a temporary dysfunction of the mesocortical and nigrostriatal dopaminergic systems that may be mediated by oxidative damage. Such perturbations might become more prominent and perhaps irreversible with a more protracted or severe stressor exposure.

Consistent with this possibility, repeated social defeat was found to exert biochemical effects similar to those of chronic CRF, but with effect of larger magnitude and more rapid onset. That is, repeated, but not a single, social defeat markedly reduced DA levels in PFC and increased DOPAC levels in both striatum and PFC 1 day following the final defeat. Moreover, repeated, but not a single, defeat reliably increased free MDA levels in both striatum and prefrontal cortex 1 day following the final defeat. Indeed, 75% of repeatedly defeated rats exhibited detectable levels of free MDA in striatum in contrast to fewer than 30% of controls. Thus, chronic exposure to a naturalistic, ecologically relevant psychological stressor elicited monoamingergic and oxidative changes in dopaminergic projection areas that were as or more pronounced than those elicited by the chronic CRF treatment. Subsequent studies are warranted to assess the enduring functional consequences of this potent, antagonistic stress regimen on behavioral measures subserved by these dopaminergic substrates.

The mesostriatal dopaminergic pathway is believed to be primarily responsible for the stereotyped behavior elicited by d-amphetamine administration and eticlopride-induced catalepsy [2]. The fact that the dopamine striatal levels were not changed in response to CRF or repeated defeat suggests that the blunted stereotypy response to amphetamine and the increased sensitivity to the cataleptic action of a D2 antagonist may be due to either a more subtle neurodegenerative change not sensitive to the

measures used in the present study, or to a rearrangement of the dopaminergic receptors in this area, such as a concomitant decrease of D1 and D2 receptors. In agreement with the receptor down-regulation hypothesis, previous studies show that chronic mild stress causes a decrease in D2-receptor binding in the nucleus accumbens accompanied by a pronounced functional subsensitivity to the rewarding and locomotor stimulant effects of D2/D3 agonist quinpirole, administered systemically or within the accumbens [21, 22]. The increased striatal DA turnover observed following chronic CRF and repeated defeat could therefore represent a compensatory response to meet increased dopaminergic demand.

The biochemical results, in particular the preferential decrease of dopamine levels in the PFC, suggest that some brain areas may be more vulnerable to the effects of chronic CRF/psychological stress. These results are in agreement with previous studies demonstrating that dopaminergic projection areas display differential sensitivity to the effects of stress [10, 12, 13, 20]. In fact, an acute stressor (tail shock) induces greater DA efflux in the PFC than in other DA projection areas, and chronic stress (exposure to cold) induces a sensitization of DA efflux in the PFC in response to tail shock [12]. Decreased PFC dopamine content also has been observed after exposure to cold water [20]. As in the present study, the increased DA content in the NAc has been shown to accompany PFC DA depletions [7, 27] (see also references within), possibly reflecting that decreased PFC dopamine causes subtle changes to functional dopamine tone in other dopamine projection areas [27].

Consistent with the notion that the PFC is more sensitive to chronic stimulation of brain stress systems than other areas, chronic CRF treatment preferentially produced changes in PFC levels of MDA at doses that did not alter striatal MDA levels. Specifically, chronic CRF produced a significant decrease in total MDA levels in the PFC 1 week following treatment. This decrease followed a marginal increase in total MDA levels one day after CRF treatment. This temporal profile of MDA levels has been interpreted previously as an induction of antioxidant functions in response to a mild, chronic oxidative insult [17]. Dopaminergic transmission in the PFC has been implicated in the working memory deficit in Parkinson's disease, depression, schizophrenia, and other disorders [9, 18], and both in animal models of Parkinson's disease as well as in humans, the onset of cognitive deficit precedes motor impairments [6, 15, 26]. Unfortunately in the present study we did not perform any cognitive

tests to investigate if the depletion of dopamine observed in the PFC was accompanied by functional behavioral deficits. Future studies are necessary to address this point.

Taken together, the current data suggest that chronic exposure to high CRF levels induces region-specific responses in DA projection areas. While these changes proved to be reversible, it is hypothesized that a stronger or more protracted CRF activation could lead to more enduring and pervasive changes in dopaminergic functioning. The observation that repeated social defeat produced short-onset, oxidative and monoaminergic changes not only in the prefrontal cortex, but also in the striatum, supports this possibility. The contribution of these processes to the pathogenesis and/or progression of disorders of the dopaminergic systems, particularly in vulnerable individuals, warrants continued study.

Specific Aim 2: To selectively breed rats for hyper- and hypo-responsiveness of the HPA axis

Experiment 2: Selective breeding of rats for high response to stress (HRS) and low response to stress (LRS) using the ACTH response to stressor exposure as the selected phenotype.

Overview: The purpose of this experiment is to develop phenotypes of hyper- and hypo-responsiveness to stress through selective breeding of the corticotropin-releasing factor (CRF) hypothalamic-pituitary-adrenal (HPA) axis response to stress. The ultimate goal of this project is to examine the role of individual vulnerability to stress in the relationship between chronic stress and neuronal pathology. Duplicate lines of rats are being selectively bred from an outbred stock of n/NIH rats based on their cumulative adrenocorticotrophic hormone (ACTH) responses to footshock stress, as described below. Rats of line 1 are referred to as HSR1 and LSR1, and line 2 rats are HSR2 and LSR2. The establishment of a second independent line allows for the validation of findings from the first line. Such duplicate breeding is an established procedure in selective breeding programs to ensure the trait specificity of the selective line, and to eliminate selection artifacts that are not related to the trait under investigation. Selection has been effective in each line as both males and females have diverged in their ACTH responses according to their intended lineage.

Subjects

Male and female breeding n/NIH outbred stock rats were obtained from the National Institutes of Health. Rats were implanted with indwelling jugular intravenous (i.v.) cannulae and allowed to recover for 2-3 days prior to testing for cumulative ACTH responses to footshock.

Selective breeding for cumulative ACTH responses to footshock

The original shock paradigm for selection utilized a 0.5 mA footshock session (1 sec shocks, 2 shocks/min, 60 min session) followed three days later by a 0.25 mA shock session. On each of the two experimental shock days, male and female rats were rank-ordered according to their total ACTH response (the sum of ACTH levels determined 0, 10, 30, 45 and 60 min following shock initiation). The rank number was averaged over the two test days to provide an overall ranking of stress response within each gender. As shown in Figures 11 and 12, male and female rats of the genetically heterogeneous n/NIH stock showed significant individual differences in their ACTH response to a mild footshock stressor prior to selection. Without regard to lineage, extreme scorers were selected and paired for breeding. Such "open" selection includes more divergent genes with each selection step and maintains increased genetic diversity relative to selection within lineage. This procedure was used to select through the third generation of the first line and the first generation of the second line.

At that time, it became clear that ACTH responses to the different intensity footshocks were not highly correlated within individual rats. Therefore, selection for subsequent generations was based on rank-ordered responses to two 0.25 mA footshock sessions. As shown in Figures 11 and 12, this shock intensity also provides a wider range of responses from which to select in the outbred population. Selective breeding has continued using these testing procedures.

Results

Divergence of HSR and LSR sub-lines

Presently, the 7th generation of Line 1 and the 5th generation of Line 2 are about to be generated, pending completion of ACTH phenotyping of their parental generation, which have been weaned. Figure 13 shows the representative divergence of ACTH responses of the offspring to the 0.25 mA footshock sessions between the initial generations of HSR and LSR sub-lines. Figure 14 shows the corresponding parents for each respective generation, selected according to their extreme ranks for ACTH responses to footshock. Figure 14 also shows that the most extreme scorers diverge with each

generation, consistent with the desired segregation and concentration of stress-responsive from non-stress-responsive genes.

Following phenotyping of the Line 2 F3 generation, new shockers (Coulbourn HO2-08 grid floor shocker controlled by a Macintosh computer; Coulbourn Instruments, Allentown, PA) were acquired and applied for testing of the Line 1 F5 generation. Using the new shockers, which were clean and therefore more efficient, ACTH responses were observed to be higher for a given shock intensity (0.25 mA; compare scales of Figures 15 and 16). Consequently, absolute values across generations in Figure 13 should be interpreted cautiously, as depicted generations were tested using the older, increasingly less efficient shockers. That is, shockers continued to elicit reliable relative differences in ACTH responses at a given point in time (and therefore interpretable differences within simultaneously tested generations). Across generations, however, absolute responses to ACTH may have declined as a result of less efficient shock delivery. Therefore, it is not clear whether HSR and/or LSR rats deviated from the n/NIH outbred stock. This question can be resolved with simultaneous comparison of selectively bred and reference unselected rats in the new shock apparatus.

Figures 15 (Line 1 F5) and 16 (Line 2 F3) depict the ACTH responses to footshock of the most recent, fully phenotyped generations from each replicate line. As can be seen, male and female HSR offspring from each line exhibit significantly greater ACTH responses to footshock stress than their LSR counterparts, confirming the success of the selective breeding program. Effect sizes tend to be larger in the F5 generation of Line 1 than in the F3 generation of Line 2, in particular for females. It is not clear whether the larger substrain differences in Line 1 F5 reflect the incremental effects of 2 generations of selection as opposed to the amplifying effects of the new shock generator.

Additional evidence of the success of the selective breeding program comes from analysis of the lineage of the most extreme scorers in the most current generation (i.e., those selected to be breeders for the subsequent generation). In the F5 generation of line 1, 6/6 of female rats and 5/6 of male rats with the greatest ACTH response to stress were descended from an HSR-HSR mating. These percentages (100% and 83%) differ significantly from the expected base rate percentages if selective breeding was ineffectual (54% [21/39], $\chi^2[1]=6.1$, $p<0.02$, and 48% [16/33], $\chi^2[1]=3.57$, $p<0.06$). Similarly, 5/6 (83%) each of female and male rats with the smallest ACTH response to stress resulted

from an LSR-LSR mating, contrasting with the corresponding expected "chance" percentages (46%, $\chi^2[1]=3.94$, $p<0.05$, and 52%, respectively, $\chi^2[1]=2.97$, $p<0.08$). Similarly, extreme scorers in males from the F3 generation of Line 2 were more likely to concord with their lineage than expected by chance (low scorers: 88% vs. expected 45%, $\chi^2[1]=7.53$, $p<0.01$; high scorers: 88% vs. expected 55%, $\chi^2[1]=4.63$, $p<0.05$). Extreme scoring females from the F3 generation of line 2 also tended to exhibit the same concordance with lineage, but these associations did not achieve statistical significance (data not shown).

Intersession consistency of ACTH responses to footshock stress

Table 5 shows the intersession consistency for male and female rats in each line, calculated as Pearson correlations. In contrast to the relative lack of correlation between ACTH responses to the two different shock intensities that were observed at the outset of selective breeding, responses to the 0.25 mA shock sessions showed good-to-excellent correlation with one another on both a continuous and rank-order basis. Correlations for the F5 generation of line 1 tended to be even larger than those from the F3 generation of line 2. Future studies can determine whether this greater association reflects use of the new shock generator or an increasing proportional contribution of genetic differences to variability in ACTH responses as a result of the additional rounds of selective breeding.

Further behavioral phenotyping of stress-responsiveness of the HSR and LSR rat lines

To determine whether the HSR and LSR sublines also differed in their behavioral responses to stressful situations, candidate breeders for the F6 generation of Line 1 were tested for their anxiety-like behavior in a 5-min defensive withdrawal test, also known as the test of situational anxiety. The defensive withdrawal test relies on the approach-avoidance conflict present in rats during exploration of novel, unprotected spaces, with anxiety-like behavior reflected as increased preference for/withdrawal into the sheltered portion of the apparatus [28]. Rats are placed in a sheltered, cylindrical chamber that is placed within a brightly lit and unsheltered, novel open field. As shown in Figure 17, both males and females of the HSR line tended to spend less time in the exposed portion of the defensive withdrawal apparatus than their LSR counterparts (*n*'s=6-7 per line), reflecting a consistent trend for increased anxiogenic-like behavior. Similarly, whereas 53% of LSR rats (7/13) emerged from the withdrawal chamber within 5 min to explore the arena, only 31% (4/13) of HSR rats did so. Although these preliminary results did not achieve statistical significance, they suggest that the

HSR and LSR sub-lines are also diverging (albeit more slowly) in their anxiogenic-like responses to stressful environments, an associated, stress-related trait not under direct experimental selection. Assessment of behavioral responses to stress will be expanded to include testing in the elevated plus maze, defensive burying and cork gnawing tests. Tests will be performed in parents following their successful breeding, so as not to disturb the breeding program and will be performed intermittently with subsequent generations of HSR and LSR rats from each line. These studies will help determine whether sub-lines are diverging on the coordinated, organismic response to stress, as intended, as opposed to the specific ACTH response to stress.

Interpretation of Results – Specific Aim 2

Replicate lines are being selectively bred for divergent ACTH responses to footshock stress. In the most current generations, lines have statistically diverged, as reflected both in central tendency and in most extreme scorers. Line 1 appears to have diverged slightly more than line 2, a finding especially evident in females, which suggests that continuing selection is worthwhile. Testifying to the robustness of the phenomenon and the reliability of assessment, significant inter-session correlations in ACTH responses to footshock are also observed. Preliminary results suggest that these rats also may be diverging in their behavioral stress responsivity. Current and future studies will focus on better characterizing the full phenotype generated by the ACTH-driven selective breeding program. Collectively, these findings confirm the effectiveness of the selective breeding program and warrant continuation of the program to further separate these replicate lines that genetically differ in their stress responsiveness. Shortly, it will be possible to examine the effects of chronic stress on neuronal pathology in these divergent, replicate lines. More generally, these lines will be invaluable to the scientific community for understanding the basis and functional importance of genetically determined individual differences in stress-responsiveness.

KEY RESEARCH ACCOMPLISHMENTS

- Proof-of-concept: Administration of ferrous citrate, an oxidizing agent, produced changes in neuropharmacological responses that reflect impaired striatal dopaminergic functioning.

- Chronic administration of CRF, a form of psychological stress, but not chronic ether stress, a physical stressor, produced transient, functional changes in responses to neuropharmacologic probes reminiscent of impaired striatal dopaminergic functioning.

- Both chronic administration of CRF and repeated defeat produced neurochemical changes consistent with increased dopamine turnover / metabolism in striatum and prefrontal cortex. In addition, both of these forms of chronic psychological stress were associated with transient tissue depletion of dopamine in prefrontal cortex, but not striatum. These findings support the hypothesis that chronic psychological stress produces sustained activation and recruitment of dopaminergic projections to these brain regions.

- Repeated defeat significantly increased free maldonialdehyde (MDA) levels, a marker of recent lipid peroxidation that is a product of oxidative stress, in both striatum and prefrontal cortex. To a lesser degree, chronic administration of CRF transiently increased total MDA levels in prefrontal cortex. These findings support the hypothesis that chronic psychological stress can increase oxidative stress in dopamine projection areas.

- Whereas the prefrontal cortex was most sensitive to the effects of chronic CRF on biochemical measures of dopaminergic functioning and oxidative stress, both striatum and prefrontal cortex were affected by repeated defeat, an extremely potent, ecologically relevant form of psychological stress.

- Replicate lines that differ in their ACTH responses to stress have been and continue to be developed. Lines have statistically diverged and show good-to-excellent intersession consistency in ACTH responses, confirming the replicability of the phenomenon.

- Preliminary results suggest that the differential responsiveness of these sub-lines to stress is general and not specific to ACTH responses to footshock.

REPORTABLE OUTCOMES:

One manuscript emanating from this work is currently under review (see Appendix; "Impairment of dopamingergic system function after chronic treatment with corticotropin-releasing factor," co-authored by Emmanuela Izzo, Pietro Paolo Sanna and George F. Koob). In addition, two additional manuscripts separately reporting results from the studies of social defeat and development of the replicate rat lines bred for differential HPA-axis responses to stressors are currently in preparation. The rat lines will be a major resource for the scientific community and can be developed further and disseminated widely.

CONCLUSIONS:

The work accomplished supports the hypothesis that brain stress systems, when chronically and profoundly activated, produces functional and oxidative changes in brain dopaminergic projection fields. This is of significance because brain dopaminergic systems are involved not only in the inititation of movement, but also in complex planning and sequencing tasks ("executive functions") as well as having a major role in motivational processes. Drugs that activate the brain dopaminergic systems are used to sustain performance in situations of fatigue (e.g., d-amphetamine) and at a minimum, one can extrapolate that a hyperresponsive brain stress system would make an individual extremely vulnerable to exhaustion and less responsive to the arousing, alerting and activating effects of dopamine system stimulants. Consequently, they would be less able to function in sustained combat missions.

The observation that repeated defeat produced more pervasive and larger magnitude changes than those elicited by chronic CRF administration indicates that naturalistic stressors have the capacity to produce functionally relevant changes in brain dopaminergic and oxidative processes. Social defeat is a stressor with obvious relevance to military concerns, as it includes the experience and expectation of antagonistic encounters, conditions directly relevant to training, combat, and prisoner-of-war situations.

The selective breeding program has been successful, producing sub-lines that statistically differ both in their central tendency and extreme ranges in terms of HPA-responsiveness to stress. As desired, preliminary experiments suggest that the lines are diverging in their global stress responsiveness, as

opposed to specifically in their ACTH response to footshock. Findings indicate that stress responsiveness in rats has a marked genetic component as lines derived from outbred n/NIH stocks diverged significantly within fewer than 3 generations. With continued breeding, the lines will be an invaluable resource for examining the basis and functional relevance of genetically determined individual differences in stress-responsiveness for varied performance and pathological outcomes. Based on studies completed during the funding period, comparison of the dopaminergic and oxidative-related effects of repeated defeat in stress hyperresponsive vs. hyporesponsive rats appears justified.

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APPENDICES:

Attached, please find the following:

1) Tables 1-5
2) Figures 1-17
3) Manuscript under review, "Impairment of dopaminergic system function after chronic treatment with corticotropin-releasing factor," co-authored by Izzo, Sanna and Koob.
4) Recent "Assurance/Certification/Declaration Care and Use of Animals" face sheet approved by the Animal Research Committee of The Scripps Research Institute.

Table 1:

Stereotypy behavior 1 day after chronic CRF treatment: median of the score (Creese-Iversen index) for each time point for each group

Time (min)	*10*	*20*	*30*	*40*	*50*	*60*	*70*	*80*	*90*	*100*	*110*	*120*	*130*	*140*	*150*	*160*	*170*	*180*
Control	3	4	4	4	4.5	5	5	5	5	4	4	3	3	3	3	3	2	2
CRF	2.5	4	4	4	4	4	4	4.5	4	4	4	3	3	3	3	2.5	2	2

Table 2:

Stereotypy behavior 1 week after chronic CRF treatment: median of the score (Creese-Iversen index) for each time point for each group

Time (min)	*10*	*20*	*30*	*40*	*50*	*60*	*70*	*80*	*90*	*100*	*110*	*120*	*130*	*140*	*150*	*160*	*170*	*180*
Control	4	4	4	4	4	4	4	4	4	4	4	3	3	3	3	3	2	2
CRF	3	4	4	4	4	4	4	4	4	4	3	3	3	3	3	3	2	2

Table 3:

Stereotypy behavior 1 month after chronic CRF treatment: median of the score (Creese-Iversen index) for each time point for each group

Time (min)	*10*	*20*	*30*	*40*	*50*	*60*	*70*	*80*	*90*	*100*	*110*	*120*	*130*	*140*	*150*	*160*	*170*	*180*
Control	3	4	4	4	5	5	4.5	5	5	5	4	4	3	3	3	2	2	2
CRF	3	4	4	4	4	4	4	4	4	4	4	4	3	3	2.5	2	2	2

Table 4:

Cataleptic-like effects of eticlopride (0.05 mg/kg SC) in male rats chronically exposed (10 days, 3 min/day) to ether or similarly-handled controls

Interval After Chronic Ether Treatment	Controls (n-6)	Ether stress (n=8)
1 day	14.8 ± 3.1	22.6 ± 10.1
1 week	25.0 ± 6.4	66.7 ± 32.1

Values represent mean ± SEM latency (sec) to remove forepaws from an elevated bar to the floor.

Table 5:

Consistency between ACTH responses to two 0.25 mA footshock sessions in selectively bred rats

	n	*r* for ACTH concentration	*r* for rank order
Line 1 F5 males	33	0.71****	0.68****
Line 1 F5 females	39	0.71****	0.72****
Line 2 F3 males	33	0.44**	0.42*
Line 2 F3 females	38	0.51***	0.57****

*$p<0.05$ vs. 0.

**$p<0.01$ vs. 0.

***$p<0.001$ vs. 0.

****$p\leq0.001$ vs. 0.

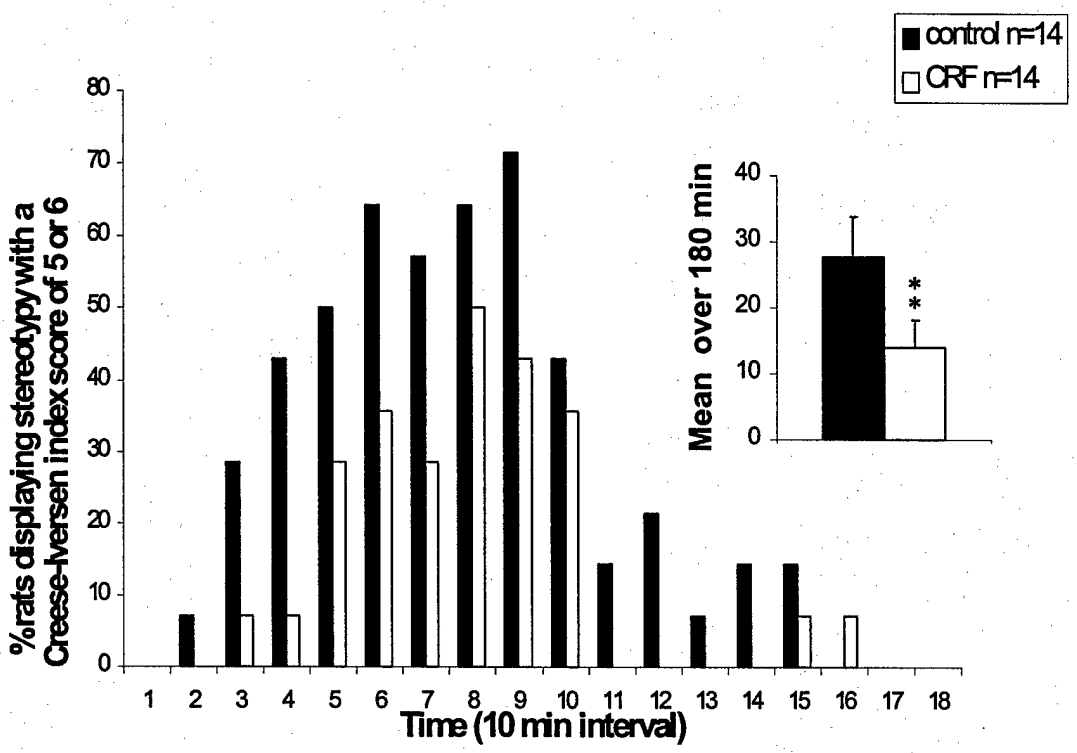

Figure 1. Stereotyped behavior induced by SC administration of 4 mg/kg d-amphetamine 1 day after chronic treatment with CRF (1 μg/day ICV for 13 days). The values represent the percentage of rats displaying stereotypy with a Creese-Iversen index score of 5 or 6 (see Body for details) at each 10 min interval during a 3-h testing session. Statistical analysis by Information Statistic revealed an overall effect of the treatment ($p<0.01$ CRF vs. control group). The inset shows the mean ± SEM of n=14 animals of the same percentage over the 3 h. (**$p<0.001$, t-test CRF vs. control).

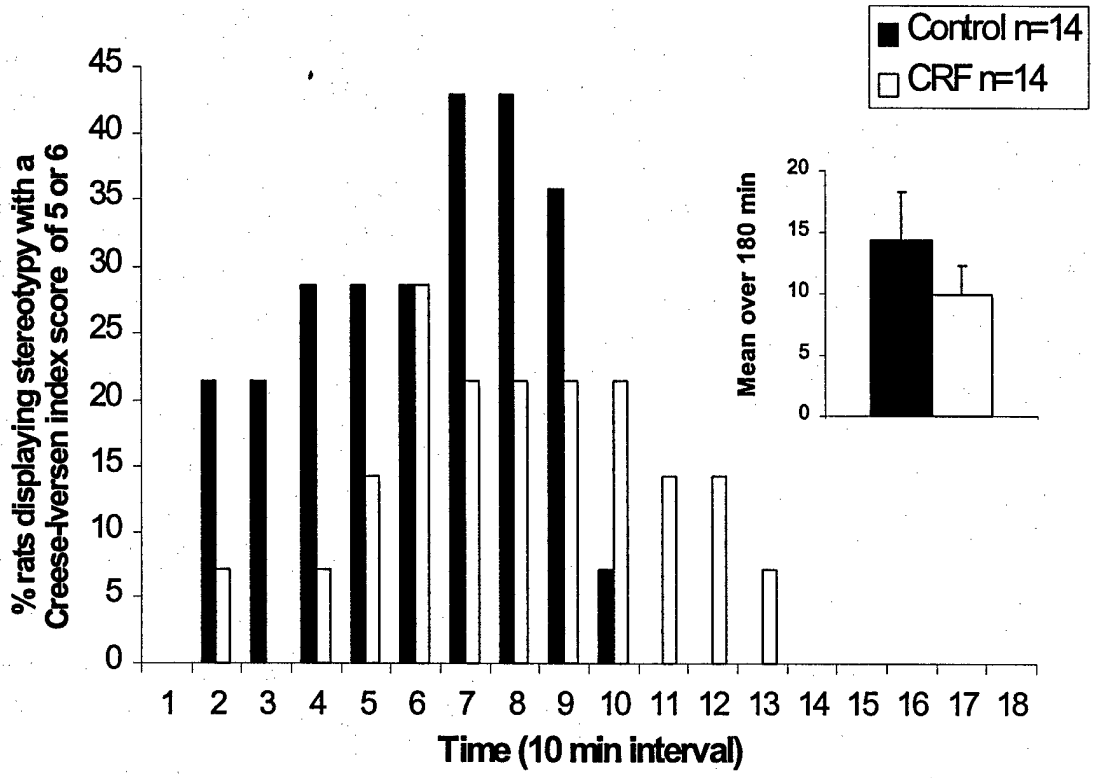

Figure 2. Stereotyped behavior induced by 4 mg/kg d-amphetamine 1 week after chronic treatment with CRF (1 μg/day ICV for 13 days). The values represent the percentage of rats displaying stereotypy with a Creese-Iversen index score of 5 or 6 (see Body for details) at each 10 min interval during a 3-h testing session. The inset shows the mean ± SEM of n=14 animals of the same percentage over the 3 h. Statistical analysis reveals a non-significant effect of treatment.

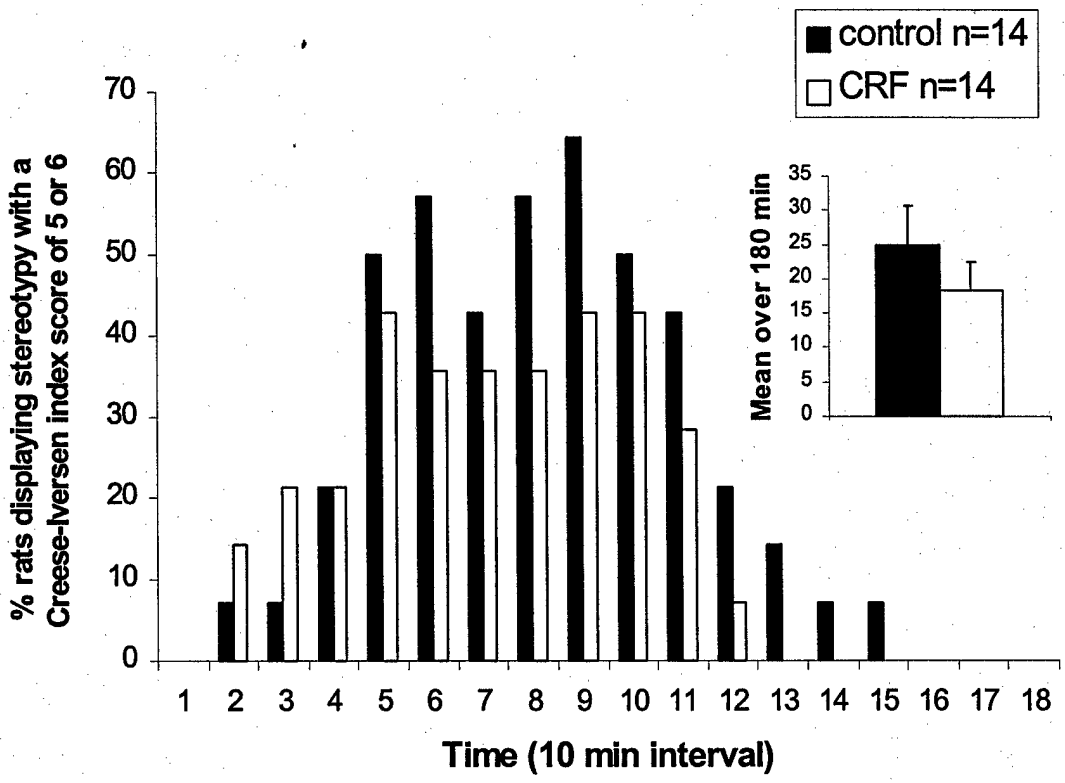

Figure 3. Stereotyped behavior induced by 4 mg/kg d-amphetamine 1 month after chronic treatment with CRF (1 μg/day ICV for 13 days). The values represent the percentage of rats displaying stereotypy with a Creese-Iversen index score 5 or 6 (see methods for details) at each 10 min interval during a 3-h testing session. The inset shows the mean ± SEM of n=14 animals of the same percentage over the 3 h. Statistical analysis reveals a non-significant effect of treatment.

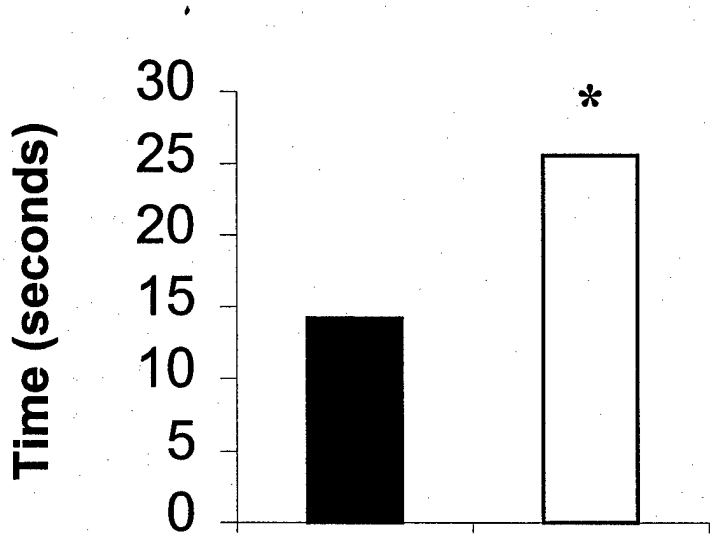

Figure 4. Catalepsy induced by SC administration of 0.05 mg/kg eticlopride 2 days after chronic treatment with CRF (1 μg/day ICV for 13 days). The values represent the median latency for controls left bar) or CRF-treated rats (right) (n's=14 per group) to reposition both forepaws from the bar to the floor (see Body for details). The test was performed 4 hours after the eticlopride injection. Statistical analysis for non-parametric measures revealed a significant effect of the chronic treatment with CRF on the cataleptic-like effect of eticlopride (*$p<0.05$ CRF vs. control, Mann Witney-U test).

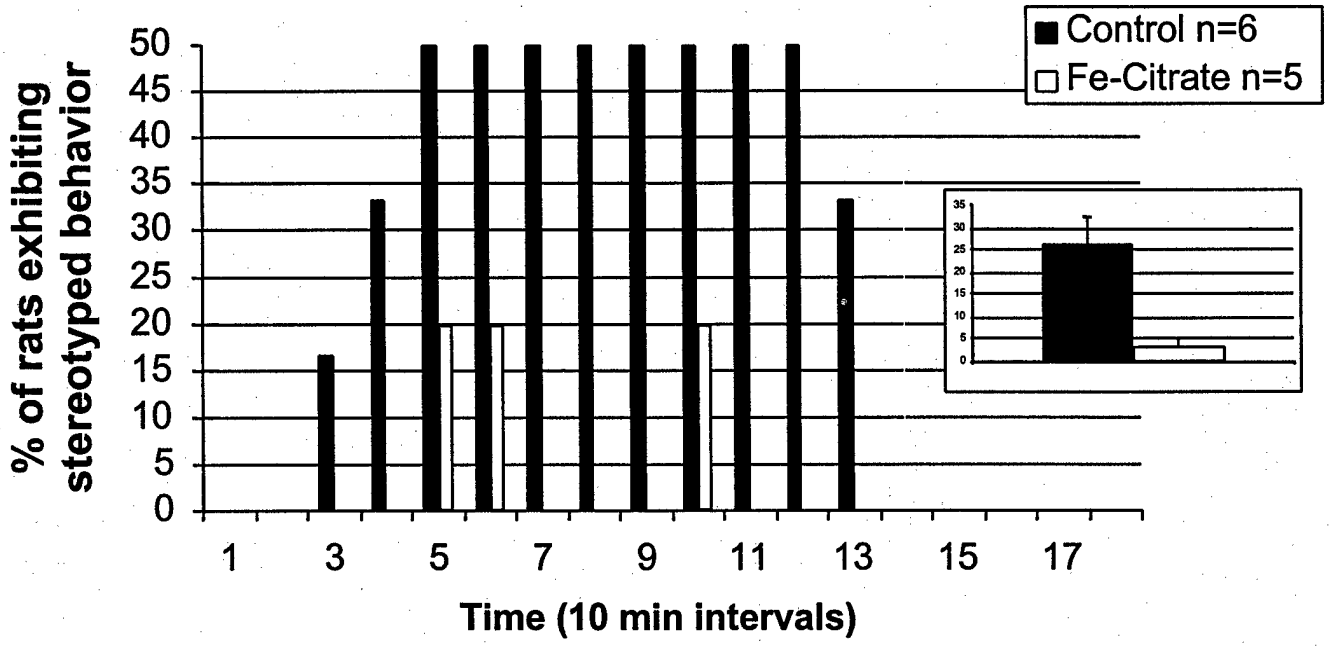

Figure 5. Effect of ICV infusion of ferrous-citrate (10 nmol) on stereotyped behavioral responses to d-amphetamine (4 mg/kg, SC). Rats were acutely injected ICV with the oxidizing agent ferrous-citrate and tested 7 days and 1 month later in the stereotypy amphetamine test. Ferrous-citrate treated rats showed a lower stereotypy response to amphetamine either 7 days or 1 month following the ferrous-citrate infusion. Results reflect testing 7 days post-iron treatment, and the inset reflect the mean % of rats exhibiting stereotyped behavior across the 180 min test session. No differences in the basal locomotor activity were present between control and iron-treated rats (not shown). Such a blunted amphetamine response suggests an impairment of the dopaminergic system and supports the notion that the dopaminergic system is especially vulnerable to oxidative insults.

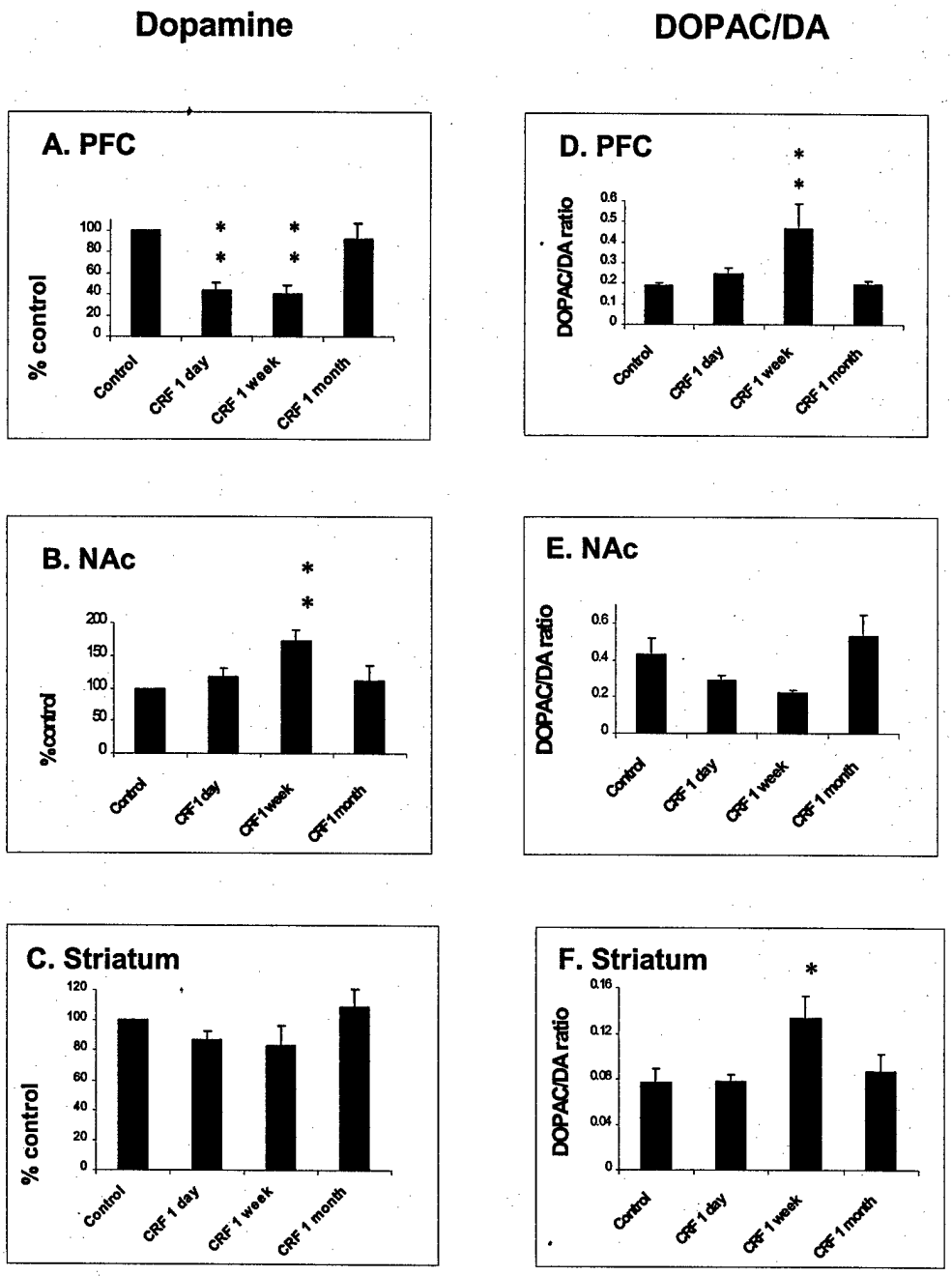

Figure 6. Panels A, B and C show dopamine tissue levels respectively in PFC, NAc and striatum after a chronic CRF treatment (1 μg/day ICV for 13 days). Values are expressed as percentage of the control average and represent the mean ± SEM of n=5-9 animals. Statistical analysis by ANOVA revealed an overall effect of the treatment in PFC and NAc (**$p < 0.005$ vs. control, Fisher's post hoc test). Panels D, E and F show DOPAC/DA ratio respectively in PFC, NAc and striatum after a chronic CRF treatment (1 μg/day ICV for 13 days). The values represent the mean ± SEM of 5-9 animals. Statistical analysis by ANOVA revealed an overall effect of the treatment in the PFC, NAc and striatum (*$p<0.01$; **$p < 0.005$ vs. control, Fisher's post hoc test).

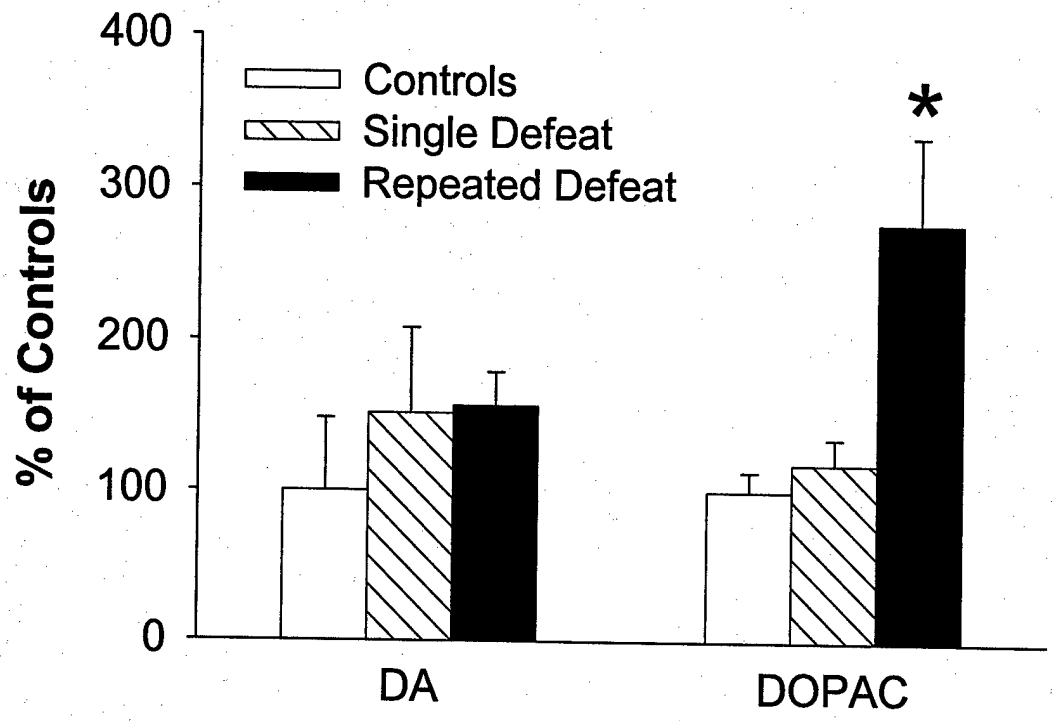

Figure 7. Dopamine and DOPAC tissue levels in striatum of animals subjected to repeated social defeat (1 defeat every other day for 21 days), acute social defeat (a single defeat on the 21st experimental day) or non-stressed controls (see Body for details of methods). Values are expressed as percentage of the control average and represent the mean ± SEM of 6-8 animals per condition one day following the final defeat session. Statistical analyses by ANOVA revealed an overall effect of the treatment in the striatum for DOPAC levels (*p<0.01 vs. both singly defeated and control rats, Fisher's post hoc test).

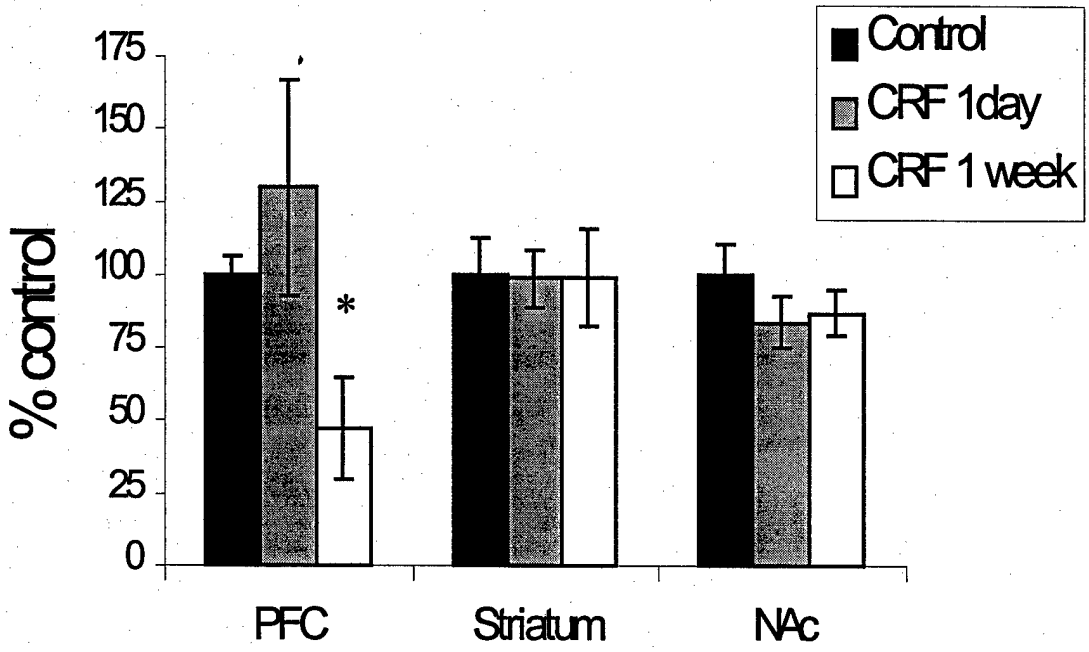

Figure 8. Malondialdehyde tissue levels in the PFC, NAc and striatum 1 day or 1 week after chronic CRF treatment (1 μg/day ICV for 13 days). The values are expressed as percentage of the control average and represent the mean ± SEM of n=3-8 animals. Statistical analysis by ANOVA revealed an overall effect of the treatment only in the PFC (*$p < 0.05$ 1 week vs. control, Fisher's post hoc test).

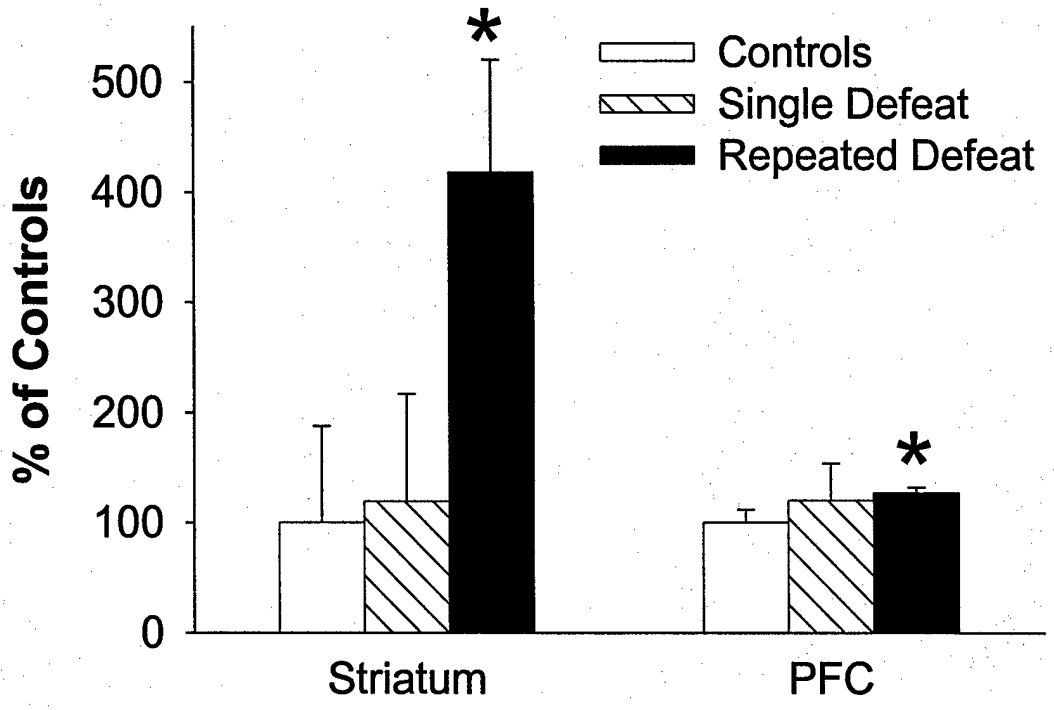

Figure 9. Malondialdehyde tissue levels in the striatum and PFC in animals subjected to repeated social defeat (1 defeat every other day for 21 days), acute social defeat (a single defeat on the 21st experimental day) or non-stressed controls (see Body for details of methods). The values are expressed as percentage of the control average and represent the mean ± SEM of 6-8 animals one day following completion of the final defeat session. Statistical analysis by ANOVA revealed an overall effect of the treatment in both striatum and the PFC (*$p<0.05$ vs. control, Fisher's post hoc test).

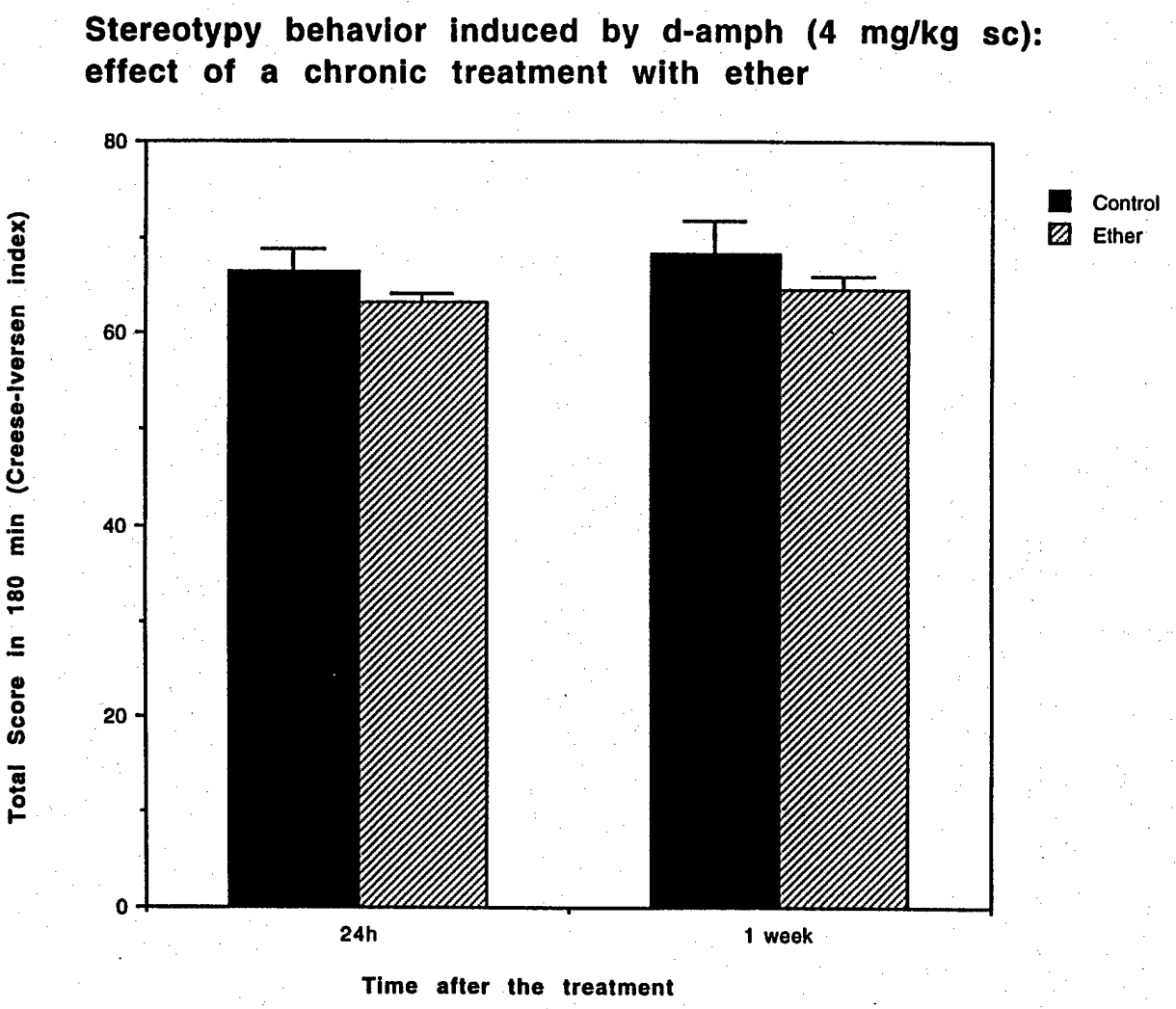

Figure 10. Effects of d-amphetamine (4 mg/kg, SC) on stereotyped behavior in chronic ether stress (3 min/day for 10 days) and control exposed male Wistar rats. Data represent the mean (± SEM) total stereotyped behavior scores using the Creese-Iversen scale (0-6) in ether-stress and control-treated subjects (n's=6-8/group) 1 day or 1 week following completion of the ether stress regimen.

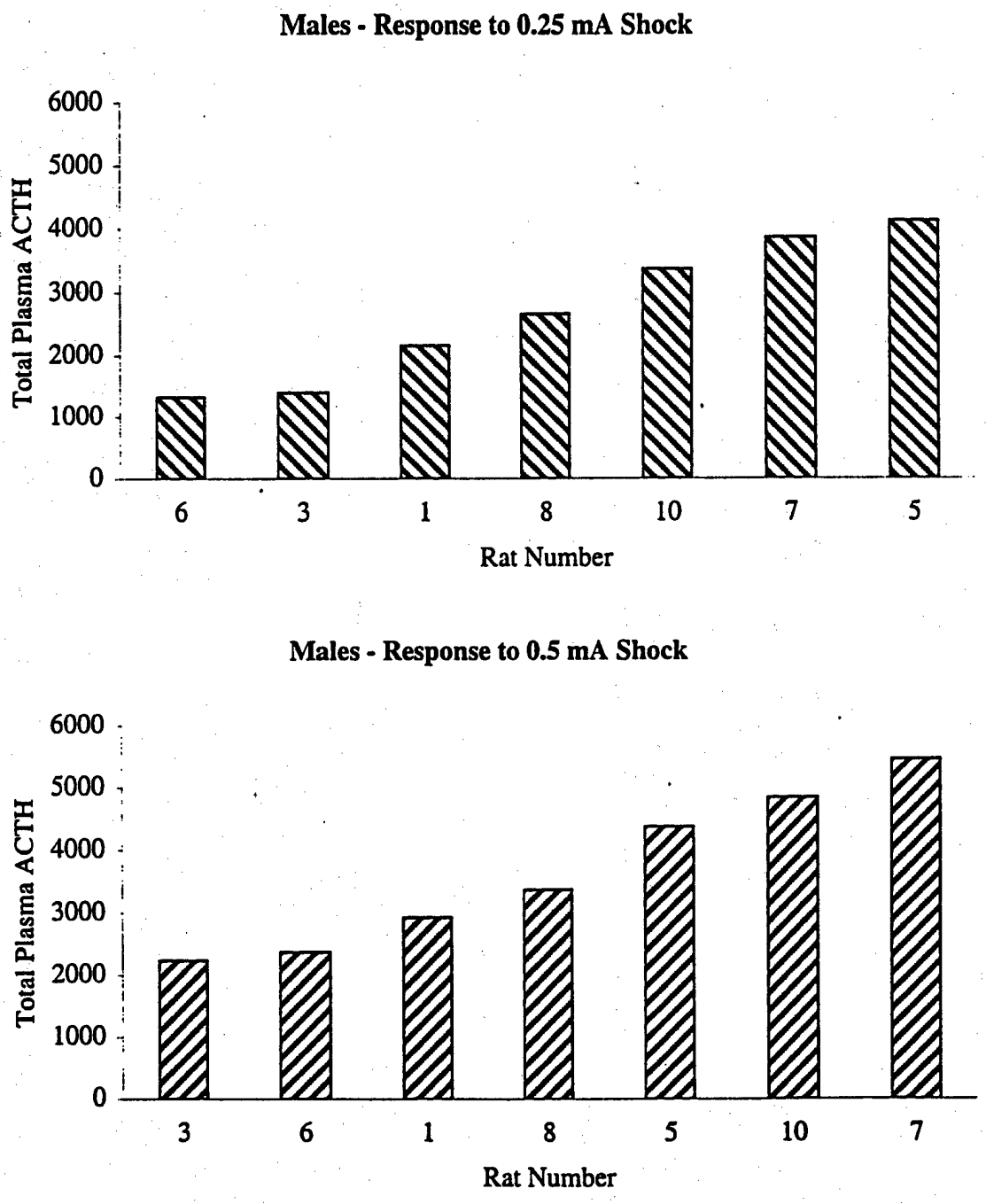

Figure 11. Cumulative plasma ACTH responses (pg) in outbred male N/NIH stock rats at two intensities of footshock stress. Values represent plasma ACTH responses by individual male rats. Males #3 and 6 were designated as low responders and #5, 7 and 10 were designated as high responders.

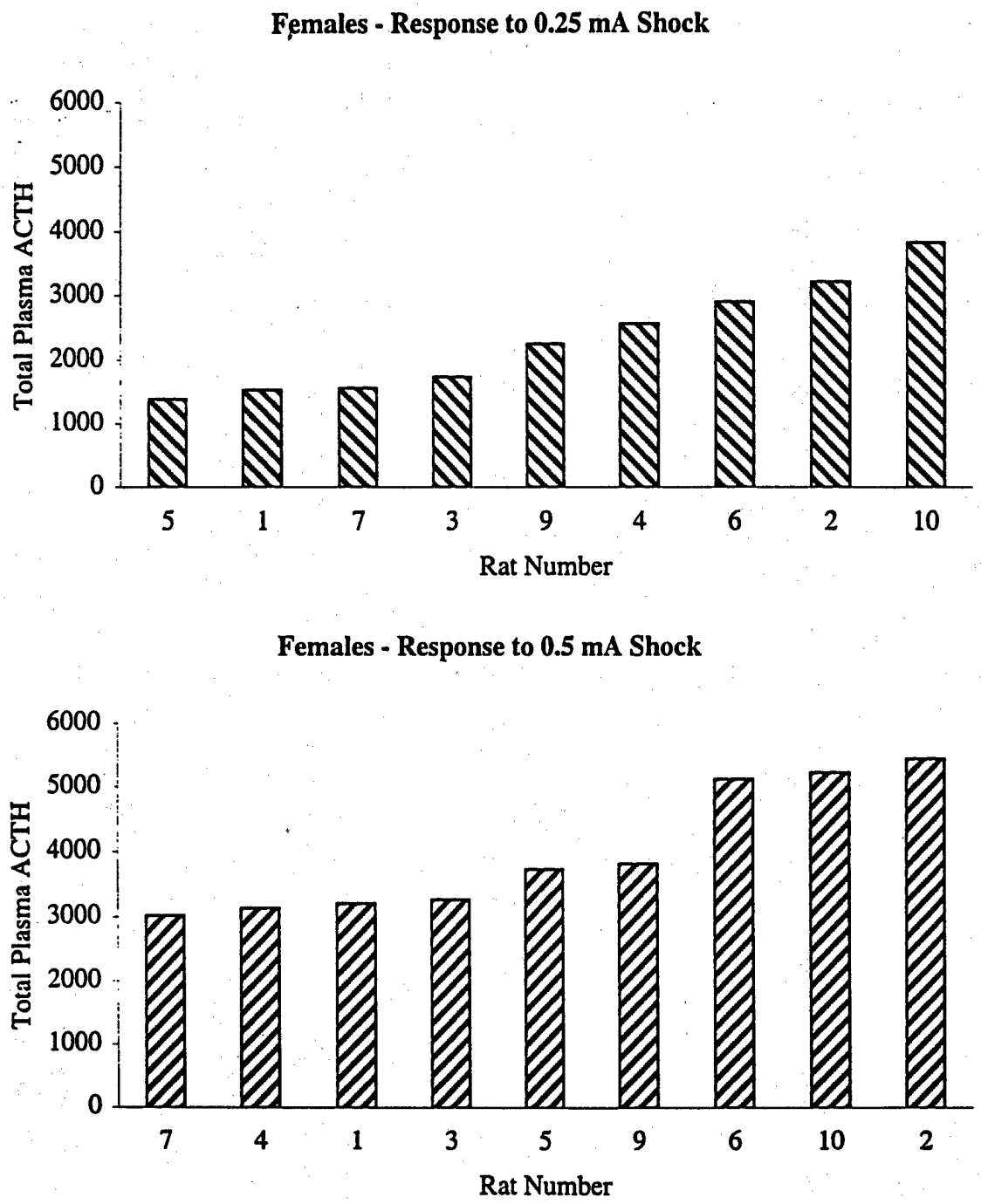

Figure 12. Cumulative plasma ACTH responses (pg) in outbred female N/NIH stock rats at two intensities of footshock stress. Values represent plasma ACTH responses by individual female rats. Females #1 and 7 were designated as low responders and #2, 6 and 10 were designated as high responders.

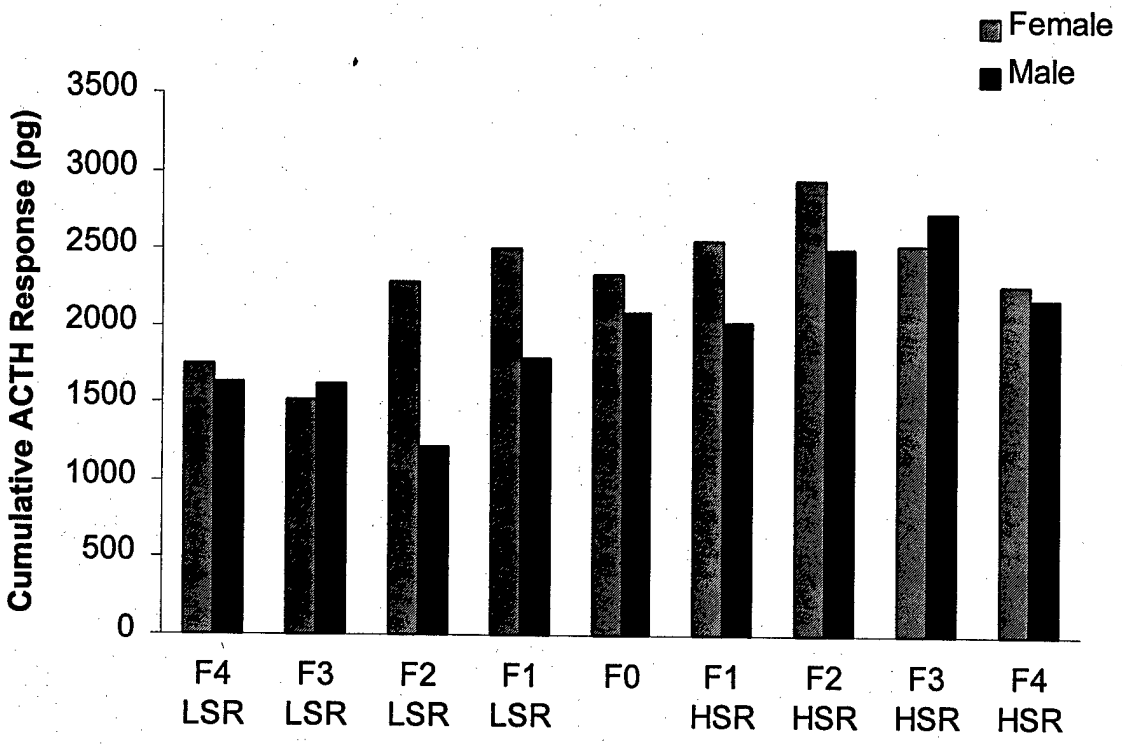

Figure 13. Selective breeding of HSR1 and LSR1 rats. Mean cumulative ACTH responses (pg) following a series of 0.25 mA footshocks in female and male rats. The base population of genetically heterogeneous n/NIH rats is denoted as F0. Selected generations 1-4 of HSR generations are shown to the right of the F0 generation, whereas LSR generations are shown to the left.

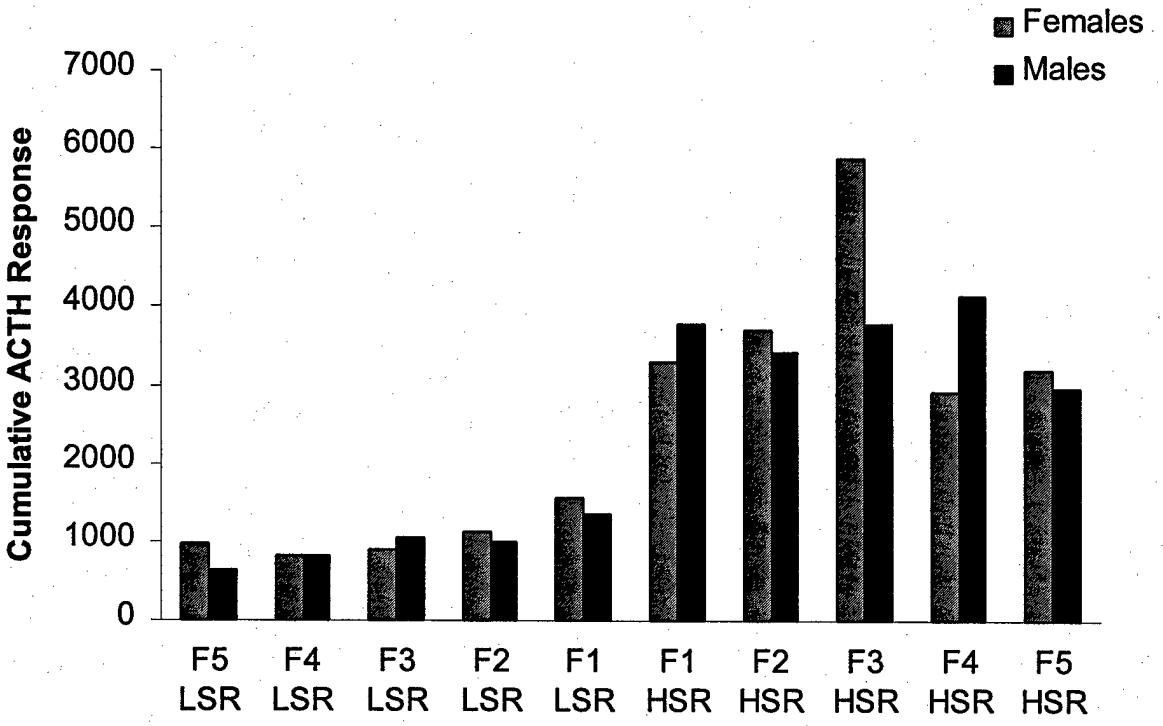

Figure 14. Breeders chosen for the continuation of HSR1 and LSR1 rat lines. Mean cumulative ACTH responses (pg) following a series of 0.25 mA footshocks in female and male rats. F1 breeders for both lines were chosen from the original n/NIH population of outbred stock rats. F2 breeders were then chosen from F1 offspring and so on. Prospective F6 breeders have been weaned and are completing ACTH phenotyping for selection and subsequent generation of the F7 offspring.

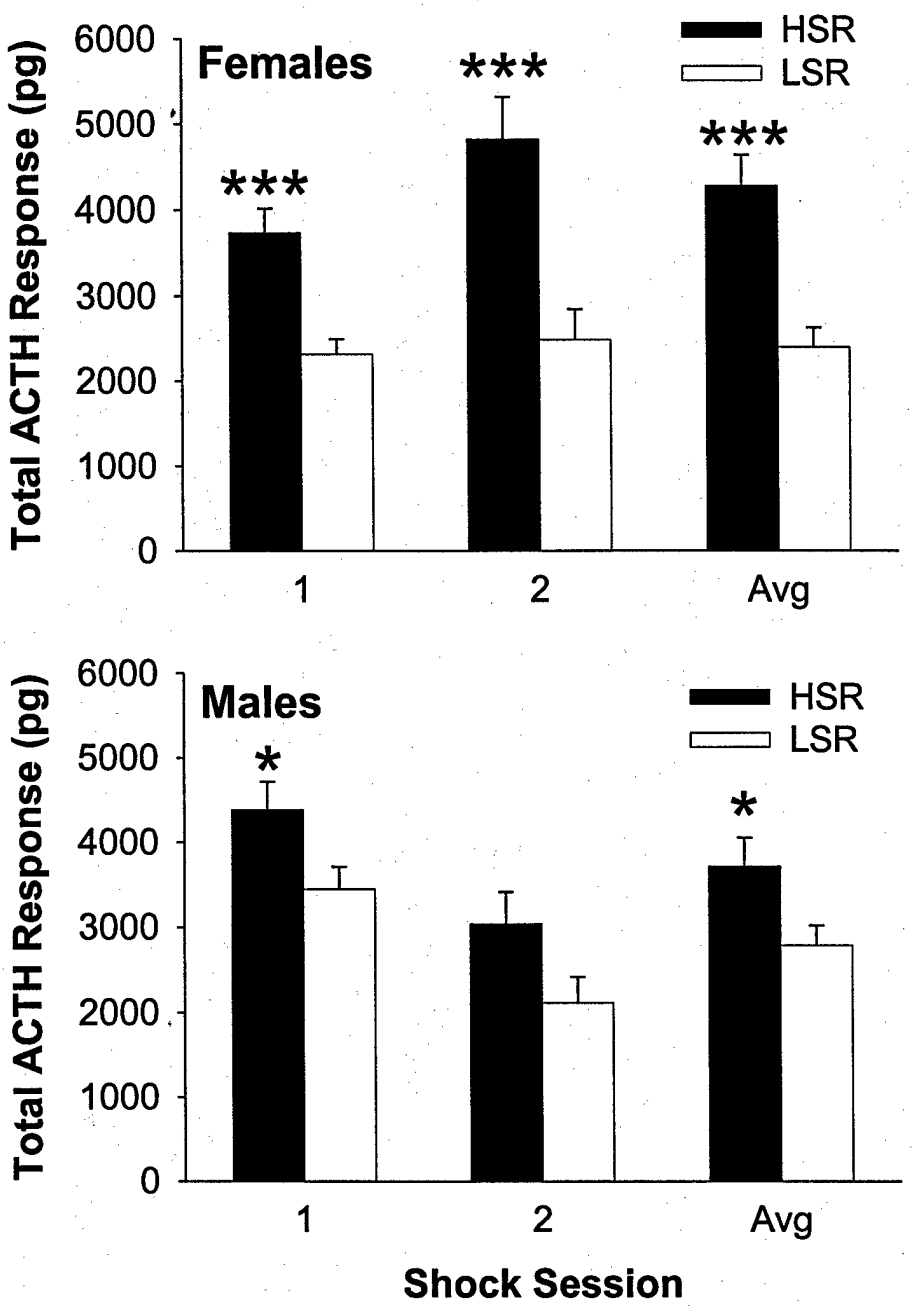

Figure 15. Mean (± SEM) total ACTH responses (pg) following a series of 0.25 mA footshocks in the female and male high-stress responsive (HSR) and low-stress responsive (LSR) F5 generation of replicate line 1 rats. Consistent with their intended phenotype, both female (*n*'s=18-21 per subline) and male HSR offspring rats (*n*'s=16-17) exhibit greater cumulative ACTH responses to footshock than their LSR counterparts (*p<0.05 , ***p<0.001 vs. LSR, Fisher's post hoc test). Please note that these ACTH data were obtained using the new shock generator. An approximate conversion to normalize these data to those obtained using the old shock generator would be to divide these values by 1.5.

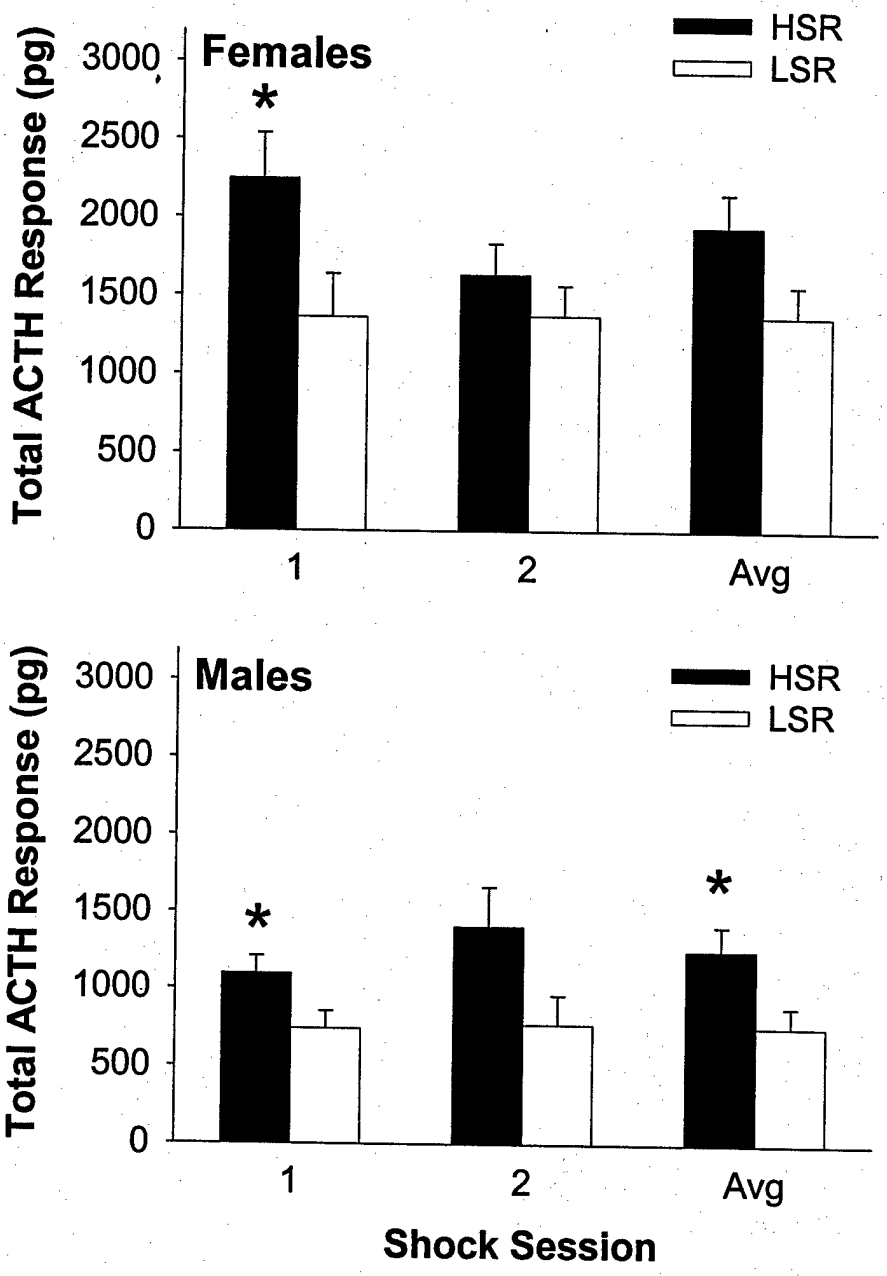

Figure 16. Mean (± SEM) total ACTH responses (pg) following a series of 0.25 mA footshocks in the female and male high-stress responsive (HSR) and low-stress responsive (LSR) F3 generation of replicate line 2 rats. Consistent with their intended phenotype, both female (*n*'s=16-22 per sub-line) and male HSR rats (*n*'s=15-18) exhibit greater cumulative ACTH responses to footshock than their LSR counterparts (*$p<0.05$ vs. LSR, Fisher's post hoc test).

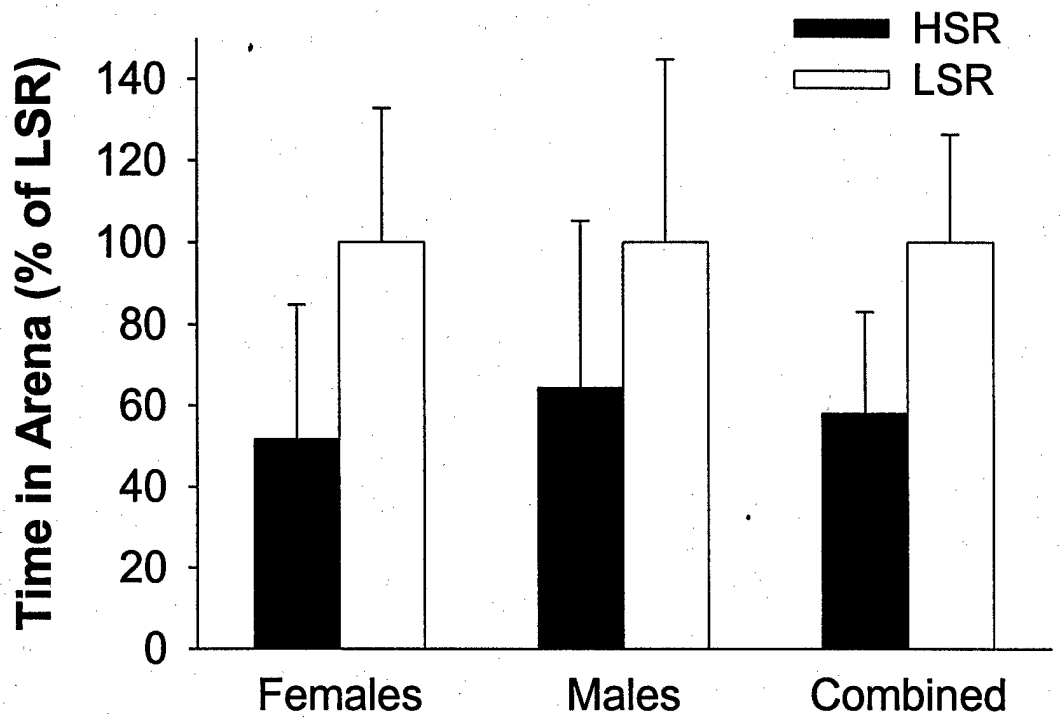

Figure 17. Mean (± SEM) time spent in the exposed arena during a 5-min defensive withdrawal test in candidate breeders for the F6 generation of the high-stress responsive (HSR) and low-stress responsive (LSR) sub-lines from replicate line 1. Values for males and females are expressed relative to the average of same-sex LSR rats to allow aggregation across sex. LSR females and males spent an average of 34 and 70 sec in the open arena during the 5-min test, respectively. Both female (*n*'s=6 per sub-line) and male HSR rats (*n*'s=7 per sub-line) tended to spend less time in the exposed portion of the defensive withdrawal apparatus than their LSR counterparts.

submitted

Impairment of dopaminergic system function after chronic treatment with corticotropin-releasing factor

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Running title: chronic stress and dopaminergic system

ABSTRACT

IZZO E., SANNA P.P., KOOB G.F. Impairment of dopaminergic system function after chronic treatment with corticotropin-releasing factor. PHARMACOL. BIOCHEM. BEHAV.

Mounting evidence suggests that chronic stress may have a detrimental effect on dopaminergic function and, in certain individuals, could contribute to the pathophysiology of central nervous system disorders like depression, schizophrenia and Parkinson's disease. Therefore, the effects of chronic elevated brain levels of corticotropin-releasing factor (CRF), a crucial mediator of the behavioral stress response, on dopaminergic function were investigated. Rats treated intracerebroventricularly (ICV) with 1 μg of CRF per day for 13 days displayed a decreased stereotypic response to d-amphetamine and an increased cataleptic response to eticlopride, consistent with decreased functional activity in the dopaminergic systems. CRF treatment also induced a transient decrease of dopamine (DA) tissue levels in the prefrontal cortex (PFC), an increase in the nucleus accumbens (NAc) and no change in the striatum (ST). A transient increase of the dihydroxyphenylacetic acid (DOPAC)/DA ratio, an indicator of dopamine turnover, also was seen in the PFC and in the striatum in CRF-treated animals. Since the dopaminergic system is very sensitive to oxidative insults, levels of malondiadyaldehyde (MDA), a membrane lipid peroxidation marker, were also measured in the same brain areas. In the PFC, we observed a decrease of MDA at 1 week after chronic CRF treatment preceded by a slight, but not statistically significant, increase in MDA. This result may indicate an activation of the antioxidant system in response to chronic stress. Taken together, these results show that a chronic hyperactivity of the CRF system leads to a transient dysfunction of the dopaminergic systems, possibly through

oxidative mechanisms, and suggest that stress could be a cofactor in the pathogenesis and/or progression of disorders of the dopaminergic systems.

KEYWORDS: chronic stress, CRF, dopamine, amphetamine stereotypy, catalepsy, oxidative stress

Stress can be defined as any condition that seriously perturbs physiological and psychological homeostasis. While the physiological and behavioral responses to stress are necessary survival mechanisms, prolonged stress can have severe repercussions varying from anxiety to impairments in learning and memory and post-traumatic stress disorder (PSTD) (Bremner,1999). Recent studies suggest that chronic stress may contribute to the pathophysiology of psychiatric disorders (e.g., depression, schizophrenia) as well as neurodegenerative diseases, such as Parkinson's disease, by affecting the dopaminergic systems (reviewed in Pani et al., 2000).

Corticotropin-releasing factor (CRF) plays a critical role in integrating stress responses, both by mediating the activation of the hypothalamic pituitary adrenal (HPA) axis and the extrahypothalamic behavioral stress response (Dunn and Berridge,1990). In the brain, activation by stress leads to a stimulation of dopaminergic neurons (Finlay and Zigmond, 1997). Therefore, it can be hypothesized that protracted activation of the brain CRF system can produce neuropathological damage in the brain dopamine (DA) system. Dopaminergic neurons are particularly susceptible to oxidative damage (Beal,1996) and DA itself causes neurotoxicity through the formation of reactive oxygen species (Stokes et al.,1999). Chronic administration of a variety of stressors, including psychological stressors, physical stress, air pollutants, and inflammatory disorders, also have been shown to induce oxidative stress both in peripheral organs and in the brain (Moller et al., 1996; Madrigal et al., 2001). Therefore, chronic stress could cause neuropathological damage to the brain DA system directly or indirectly through oxidative mechanisms.

To test this hypothesis, we investigated the effect of chronic intracerebralventricular (ICV) CRF administration on d-amphetamine-induced stereotypic behavior and on catalepsy induced by the DA D_2 antagonist eticlopride. These behavioral tests have been shown to be very sensitive to impairment of the

function of the midbrain DA system (Amalric and Koob, 1987, 1993). In addition, we measured tissue levels of DA and dihydroxyphenylacetic acid (DOPAC) in the primary dopaminergic projection areas, such as the prefrontal cortex (PFC), striatum (ST) and nucleus accumbens (NAc). Finally, malondialdehyde (MDA) levels (a product of membrane lipid peroxidation) were measured in the same brain areas as a marker of oxidative damage.

METHODS

Subjects

Fifty-four male Wistar rats (Charles River, Kingston NY), weighing 200-225 g at the start of the experiment were used. Twenty-eight rats were used for behavioral experiments and 26 for biochemical assay. Rats were housed 3 per cage and provided with ad libitum access to food and water and maintained on a 12-h light-dark cycle (lights on 7:00am-7:00pm).

ICV surgery

For stereotaxic surgery, rats were anesthetized under chronic halothane vapor (1.0-1.5%) and placed in a Kopf stereotaxic instrument (Kopf Instrument, Tujunga, CA). A 7 mm stainless steel guide cannula (23 gauge) was secured to the skull with three stainless steel screws and Silux dental cement. The coordinates were: AP, + 0.6 mm from bregma; L, $\pm$ 2.0 mm from the midline; DV, -3.2 mm from the skull surface, with the incisor bar 5 mm above the inter-aural line (Pellegrino et al, 1979). An 8.5 mm stylet was placed into the cannula and the rats were allowed 7 days to recover from surgery before treatment.

Drugs and treatments

Rat/Human CRF (Rivier et al., 1983) was kindly provided by Dr J. Rivier (Clayton Foundation for Peptide Biology, The Salk Institute). For ICV injections, CRF was dissolved in isotonic saline and injected by gravity. The stylet was removed from the guide cannula and an 8.5 mm (30 gauge) stainless steel injector connected to about 70 cm calibrated PE 10 tubing was inserted. The plastic tubing then was raised above the head of the rat until flow began. Five microliters of either saline or CRF (1μg/5μl solution) were infused over an approximately 60 s period. In order to prevent efflux, the injector was left in place for an additional 30 s before the stylet again was placed in the guide cannula. The rats were injected after 7 days recovery with 1 μg/day of CRF for 13 days. The control group received the same volume of saline. Rats were not restarined during ICV injections. d-Amphetamine sulfate was obtained from Sigma Chemical Co. (St. Louis, MO) and was dissolved in isotonic saline. Eticlopride was obtained from RBI (Natick, MA)

and was dissolved in saline. All drugs were injected subcutaneously (SC) in a volume of 1.0 ml/kg of body weight.

Apparatus

A bank of 16 photocell cages was used to measure locomotor activity and stereotypic behavior. Each cage measured 20x25x36 cm, and was made of wire mesh. Two infrared photocell beams were situated across the long axis 2cm above the floor. Interruption of a beam was registered by a computer situated in an adjoining room. Background noise was provided by a white noise generator.

Behavioral procedure

d-Amphetamine-induced stereotypic behavior

Rats (14 saline and 14 CRF treated) were habituated to the photocell cages for three hours for two consecutive days prior to the experiment day in order to overcome the potentially stressful nature of a novel environment. At different time points: 1 day, 1 week and 1 month after chronic ICV treatment, the rats were placed in the locomotor activity cages for 90 min and then injected SC with 4.0 mg/kg d-amphetamine and stereotypic behavior was rated for 180 min after the injection. Locomotor activity was also recorded but data are not shown. During the 3-h test each rat was observed every 10 min for about 10 s. Stereotypic behavior was rated according to the Creese and Iversen (1973) rating scale. This scale rates the intensity of stereotypy on a 7-point scale. The scores are defined as follows:

0: asleep or stationary

1: active

2: predominantly active, bursts of stereotyped sniffing or rearing

3: stereotyped activity, sniffing along fixed path of cage

4: stereotyped sniffing or rearing maintained in one location

5: stereotyped behavior in one location with bursts of gnawing or licking

6: continual gnawing or licking of cage bars

Behavior was rated by one observer blind to the rats' experimental treatment.

Catalepsy test

Catalepsy was measured using the bar test according to Pulvirenti and Koob (1993). Rats were injected with the DA D-2 antagonist eticlopride (0.05 mg/kg SC). Four hours after the injection both their forepaws were placed on a bar 9 cm from the floor. The time elapsed until the rats repositioned both forepaws on the floor was recorded by an experimenter blind to the treatment condition. A cut off of 5 min per observation was used. Since repeated injection of D_2 antagonists induces sensitization to the cataleptic effect of the drug (Barnes et al, 1990), rats were tested only at one time point, 2 days after chronic CRF or saline treatment and 1 day after having been tested for d-amphetamine stereotypy. Preliminary experiments demonstrated that exposure to amphetamine 24 hours earlier does not interfere with the cataleptic effect of eticlopride.

Biochemical measures

Three groups of animals were sacrificed at 1 day, 1 week and 1 month after 13 days of treatment with CRF (n=17) or saline (n=9). Rats were anesthetized with CO_2 and the brain was quickly removed and dissected using a wire brain slicer. Prefrontal cortex (PFC), striatum (ST) and nucleus accumbens (NAc) were collected and promptly frozen on dry ice. For each rat one side of the brain areas were used for dopamine and DOPAC levels determination and the controlateral side for MDA assay. Brain tissue was stored at –80°C until analysis.

Analysis of total tissue DA and DOPAC levels

Tissue samples of each region were placed into tared 1.5 ml eppendorf tubes, and the weight of tissue in each tube was determined. One ml of 0.1N perchloric acid containing 50 nM methylserotonin as an internal standard was added to each tube. The tissue then was disrupted ultrasonically and the tubes were centrifuged to pellet all particulate matter. Aliquots of 100 µl of the supernatant were analyzed for DA and DOPAC. Concentrations of DA and DOPAC were determined with a microbore HPLC system

equipped with a Spherisorb C-18 column (100 x 1 mm, 3 μm spheres) using a mobile phase composed of a 54 mM dibasic sodium phosphate buffer with 12% methanol (v/v), 0.2 mM EDTA, 0.9 mM 1-decanesulfonic acid and 4.9 mM triethylamine, at a final apparent pH of 4.8, pumped at 25 μl/min by an ISCO model 500D syringe pump. The monoamines were detected using a glassy carbon electrode set at +700 mV vs. Ag/AgCl by an amperometric detector (Princeton Applied Research model 400). The detection limit for each monoamine was approximately 0.2 nM.

Malondialdehyde assay

The tissue was homogenized in 10% w/v in phosphate-buffered saline (PBS) containing butylated-hydroxytoluene (BHT) (5 mM). Malondialdehyde (MDA) total levels were determined using a colorimetric assay (Bioxytech MDA-586 from OXIS International, Portland, OR). With this method N-methyl-2-phenylndole is allowed to react with malondialdehyde at 45°C. One molecule of MDA reacts with 2 molecules of N-methyl-2-phenylndole to yield a stable chromophore with maximal absorbance at 586 nm. MDA measurements were performed on extracts of prefrontal cortex (PFC), striatum (ST) and nucleus accumbens (NAc). MDA concentrations are expressed as μmoles of MDA per mg of total proteins.

Statistical analysis

Stereotypy ratings were analyzed using the information statistic (Kullback 1968; Robbins 1977). This statistical analysis is analogous to X^2, but is not constrained by small-cell frequencies. Stereotyped behavior rating was analyzed in two ways. First at each of the 18 time intervals the number of animals that displayed a score of 5 or 6 of the Creese and Iversen index (1973) was processed and these 18 2I values were added to give a total 2I. For the analysis of the mean of the percentage of rats displaying a score of 5 or 6 over the 3 hours, a paired Student's t-test was used. Catalepsy was analyzed using the Mann Withney-U test for non-parametric measures. Biochemical data were analyzed using a one-way factor analysis of variance (ANOVA). Individual means comparisons for the main treatment effects of the biochemical data were analyzed by using a Fisher's post-hoc test.

RESULTS

Effect of chronic CRF treatment on stereotypic behavior induced by d-amphetamine

Chronic CRF treatment (1 μg/day ICV for 13 days) reduced the stereotypic behavior induced by d-amphetamine at 1 day after the treatment. Figure 1 shows the percentage of the rats displaying a stereotypic behavior rated 5 or 6 over the 3-h test period at 1 day after the CRF chronic treatment. This measure reached a maximum level at 80-90 minutes after amphetamine injection (4mg/kg SC) and then decreased to a minimum three hours later. The overall information statistic was significant ($2I=45.31$, $df=1,18$ $p<0.01$) and also, the mean of the same values for the 3-h test was significantly lower in the CRF group compared to the control group ($p<0.001$ paired Student's t-test). The stereotypic response to d-amphetamine at 1 week did not differ significantly between groups, although there was a trend to lower values in the CRF-group (Figure 2), finally no differences were present at 1 month after the treatment (Figure 3). Tables 1, 2 and 3 show the median of the value of all scores for each time point. Locomotor activity was recorded during the 90 minutes of habituation and during the 3-hr testing. No differences were observed between groups at all time points (data not shown).

Effect of chronic CRF treatment on catalepsy induced by eticlopride

Chronic CRF treatment (1 μg/day ICV for 13 days) increased the cataleptic effect of 0.05 mg/kg eticlopride, a D2 antagonist, 2 days after the treatment (Figure 4) ($p<0.05$ Mann Witney-U test for non-parametric measure treated vs control rats). Because repeated injection of D_2 antagonists induces sensitization to the cataleptic effect of the drug (Barnes et al, 1990) rats were tested only at one time point, 2 days after the chronic ICV CRF or saline treatment and 1 day after having been tested for d-amphetamine stereotypy. Preliminary experiments assessed that a previous (24 hours) exposure to amphetamine does not interfere with the cataleptic effect of eticlopride (data not shown).

Effect of chronic CRF on DA and DOPAC levels

As shown in Figure 5A, tissue DA levels in the PFC were significantly lower at 1 day and 1 week after the chronic CRF treatment when compared to control animals (overall one-way ANOVA $F(3,21)=7.3$ $p<0.005$; Fisher's post-hoc test $p<0.005$ 1 day and 1 week vs control). DA levels returned to baseline one month after CRF treatment. In the NAc (Figure 5B) DA levels were significantly higher at 1 week after the treatment (overall one-way ANOVA $F(3,22)=4.6$ $p<0.05$; Fisher's post-hoc test $p<0.005$ 1 week vs control). No significant differences in DA levels were found in the striatum at all time points (Figure 5C). The DOPAC/DA ratios were significantly increased at 1 week after the treatment both in PFC (overall one-way ANOVA $F(3,21)=5.8$ $p<0.005$; Fisher's post-hoc test $p<0.005$ 1 week vs control) and striatum (overall one-way ANOVA $F(3,22)=3.7$, $p<0.05$; Fisher's post-hoc test $p<0.01$ 1 week vs control) (Figure 5E and 5F). One-way ANOVA reveals an overall effect of the treatment in the NAc on DOPAC/DA ratio [$F(3,23)=3.12$, $p<0.05$] but no significant effect in post-hoc analysis (Figure 5D). All the changes were transient, and values returned to baseline levels one month after chronic CRF treatment and were in agreement with the behavioral data.

Effect of chronic CRF on malondialdehyde levels

A significant decrease of MDA levels was observed in the PFC (Figure 6) at 1 week after chronic CRF treatment when compared to the control group (one-way ANOVA $F(2,12)=5.4$ $p<0.01$; Fisher's post-hoc test $p<0.05$ 1 week vs control). Such a decrease was preceded by a non-significant increase at one day (Figure 6). There was no significant effect of CRF chronic treatment on malondialdehyde levels in the NAc or striatum at all time points.

DISCUSSION

The results of the present study show that chronic CRF treatment (1μg/day for 13 days) produces a lower stereotypic response to d-amphetamine and increased eticlopride-induced catalepsy, at day 1 and day 2 respectively, after the treatment, consistent with impaired striatal dopaminergic

function. The biochemical data show that the dopamine levels were decreased in the PFC at 1 day and 1 week after the treatment, increased in the NAc (at 1 week) and unchanged in the striatum . An increased DA turnover both in the striatum and in the PFC was observed only at 1 week after the CRF treatment.

Acutely, low doses of CRF (0.02-0.1 μg) have been shown to potentiate d-amphetamine-induced stereotypic behavior (Cole and Koob, 1989), and acute stress induces increased DA release in dopaminergic projection areas (Castro and Zigmond, 2001; Finlay and Zigmond, 1997; Gresch et al., 1994; Mizoguchi et al. 2000). It is therefore possible that chronic administration of CRF could lead to a persistent activation of the dopaminergic system. Our behavioral data suggest that this protracted activation of the CRF system causes a transient impairment of dopaminergic function early after the chronic stimulation.

The mesostriatal dopaminergic pathway is believed to be primarily responsible for the stereotypic behavior elicited by d-amphetamine administration and eticlopride-induced catalepsy (Dickson et al, 1994; Amalric and Koob, 1993). The fact that the dopamine striatal levels are not changed suggests that the lower stereotypy response to amphetamine and the increased sensitivity to the cataleptic action of a D2 antagonist may be due to either a more subtle neurodegenerative change not sensitive to the measures used in the present study, or to a rearrangement of the dopaminergic receptors in this area, such as a concomitant decrease of D1 and D2 receptors. In agreement with receptor down regulation hypothesis previous studies show that chronic mild stress causes a decrease in D2-receptor binding in the nucleus accumbens (Willner et al 1991, Papp et al 1994) accompanied by a pronounced functional subsensitivity to the rewarding and locomotor stimulant effects of D2/D3 agonist quinpirole, administered systemically or within the accumbens (Papp et al, 1993). The increased striatal DA

turnover observed at one week after CRF treatment, when behavioral signs had subsided, could therefore represent a compensatory response to cope to an increased dopaminergic demand.

The biochemical results, in particular the decrease of dopamine levels, suggest that some brain areas like PFC are more vulnerable then others (striatum) to the chronic CRF. These results are in agreement with previous studies demonstrating that dopaminergic projection areas display differential sensitivity to the effects of stress (Horger and Roth, 1996, Finlay and Zigmond, 1997; Gresch et al., 1994; Mizoguchi et al. 2000). In fact, an acute stressor (tail shock) induces higher DA efflux in the PFC than in other DA projection areas, and chronic stress (exposure to cold) induces a sensitization of DA efflux in the PFC in response to tail shock (Gresch et al., 1994). Decreased PFC dopamine content also was observed after exposure to cold water (Mizoguchi et al. 2000). As in the present study, the increased DA content in the NAc have been shown to accompany PFC DA depletions (Simon et al 1988; Espejo and Miñano, 2001, and references within). Nevertheless, it should be noted that such increase in NAc dopamine levels is present only at 1 week and not at 1 day. We are not able to explain this temporal discrepancy, it is possible that the NAc has a delayed response to changes in PFC. However is well documented that decrease in PFC dopamine cause subtly changes to functional dopamine tone in other dopamine projection areas (Simon et al 1988)

Consistent with the notion that the PFC is more sensitive to chronic stimulation of the CRF system then other areas, a significant decrease in the level of MDA, a marker of lipid peroxidation (Madrigal et al, 2001; Liu et al., 2000), was detected at 1 week after chronic CRF treatment in the PFC following a non-statistically significant increase one day after CRF treatment. Such a pattern of change in malondialdehyde level has been interpreted previously as a sign of the induction of the antioxidant system in response to a mild chronic oxidative insult (Liu et al, 2000). Dopaminergic transmission in the PFC has been implicated in the working memory deficit in Parkinson's disease, depression, schizophrenia, and other disorders (Mattay et al, 2002; Fibiger, 1995) and both in animal models of

Parkinson's disease as well as in humans, the onset of cognitive deficit precedes motor impairments (Schneider and Pope-Coleman, 1995; Dubois and Pillon, 1997; Kulisevsky, 2000). Unfortunately in the present study we did not perform any cognitive tests to investigate if the depletion of dopamine observed in the PFC was accompanied by functional behavioral deficits. Future studies are necessary to address this point.

Taken together the current data suggest that chronic exposure to high CRF levels induces region-specific responses in DA projection areas. While these changes proved to be reversible, it is possible that a stronger or more protracted CRF activation could contribute to the pathogenesis and/or progression of disorders of the dopaminergic systems, particularly in vulnerable individuals.

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LEGEND TO FIGURES

Figure 1: Stereotypic behavior induced by SC administration of 4 mg/kg d-amphetamine 1 day after chronic treatment with CRF (1 μg/day ICV for 13 days). The values represent the percentage of rats displaying stereotypy with a Creese-Iversen index score of 5 or 6 (see methods for details) at each 10 min interval during a 3-h testing session. Statistical analysis by Information Statistic revealed an overall effect of the treatment ($p<0.01$ CRF vs control group). The insert shows the mean ± SEM of n=14 animals of the same percentage over the 3 h. (**$p<0.001$ paired t-test CRF vs control).

Figure 2: Stereotypic behavior induced by 4 mg/kg d-amphetamine 1 week after chronic treatment with CRF (1 μg/day ICV for 13 days). The values represent the percentage of rats displaying stereotypy with a Creese-Iversen index score of 5 or 6 (see methods for details) at each 10 min interval during a 3-h testing session. The insert shows the mean ± SEM of n=14 animals of the same percentage over the 3 h. Statistical analysis reveals a non-significant effect of treatment.

Figure 3: Stereotypic behavior induced by 4 mg/kg d-amphetamine 1 month after chronic treatment with CRF (1 μg/day ICV for 13 days). The values represent the percentage of rats displaying stereotypy with a Creese-Iversen index score 5 or 6 (see methods for details) at each 10 min interval during a 3-h testing session. The insert shows the mean ± SEM of n=14 animals of the same percentage over the 3 h. Statistical analysis reveals a non-significant effect of treatment.

Figure 4: Catalepsy induced by SC administration of 0.05 mg/kg eticlopride 2 days after chronic treatment with CRF (1 μg/day ICV for 13 days). The values represent the median of n=14 animals of the time spent by rats to reposition both forepaws from the bar to the floor (see methods for details). The test was performed 4 hours after the eticlopride injection. Statistical analysis for non-parametric measures revealed a significant effect of the chronic treatment with CRF on the cataleptic efficiency of eticlopride (*$p<0.05$ CRF vs control, Mann Witney-U test).

Figure 5: Panel A, B and C show dopamine tissue levels respectively in PFC, NAc and striatum after a chronic CRF treatment (1 μg/day ICV for 13 days). Values are expressed as percentage of the control average and represent the mean ± SEM of n=5-9 animals. Statistical analysis by ANOVA revealed an overall effect of the treatment in PFC and NAc (**$p < 0.005$ vs control, Fisher's post hoc test).

Panels D, E and F show DOPAC/DA ratio respectively in PFC, NAc and striatum after a chronic CRF treatment (1 μg/day ICV for 13 days). The values represents the mean ± SEM of n=5-9 animals. Statistical analysis by ANOVA revealed an overall effect of the treatment in the PFC, NAc and striatum (*$p<0.01$; **$p < 0.005$ vs control, Fisher's post hoc test).

Figure 6: Malondialdehyde tissue levels in the PFC, NAc and striatum 1 day and 1 week after chronic CRF treatment (1 μg/day ICV for 13 days). The values are expressed as percentage of the control average and represent the mean ± SEM of n=3-8 animals. Statistical analysis by ANOVA revealed an overall effect of the treatment only in the PFC (*$p < 0.05$ 1 week vs control, Fisher's post hoc test).

Table 1:

Stereotypy behavior 1 day after chronic treatment: median of the score (Creese-Iversen index) for each time point for each group

Time (min)	*10*	*20*	*30*	*40*	*50*	*60*	*70*	*80*	*90*	*100*	*110*	*120*	*130*	*140*	*150*	*160*	*170*	*180*
Control	3	4	4	4	4.5	5	5	5	5	4	4	3	3	3	3	3	2	2
CRF	2.5	4	4	4	4	4	4	4.5	4	4	4	3	3	3	3	2.5	2	2

Table 2:

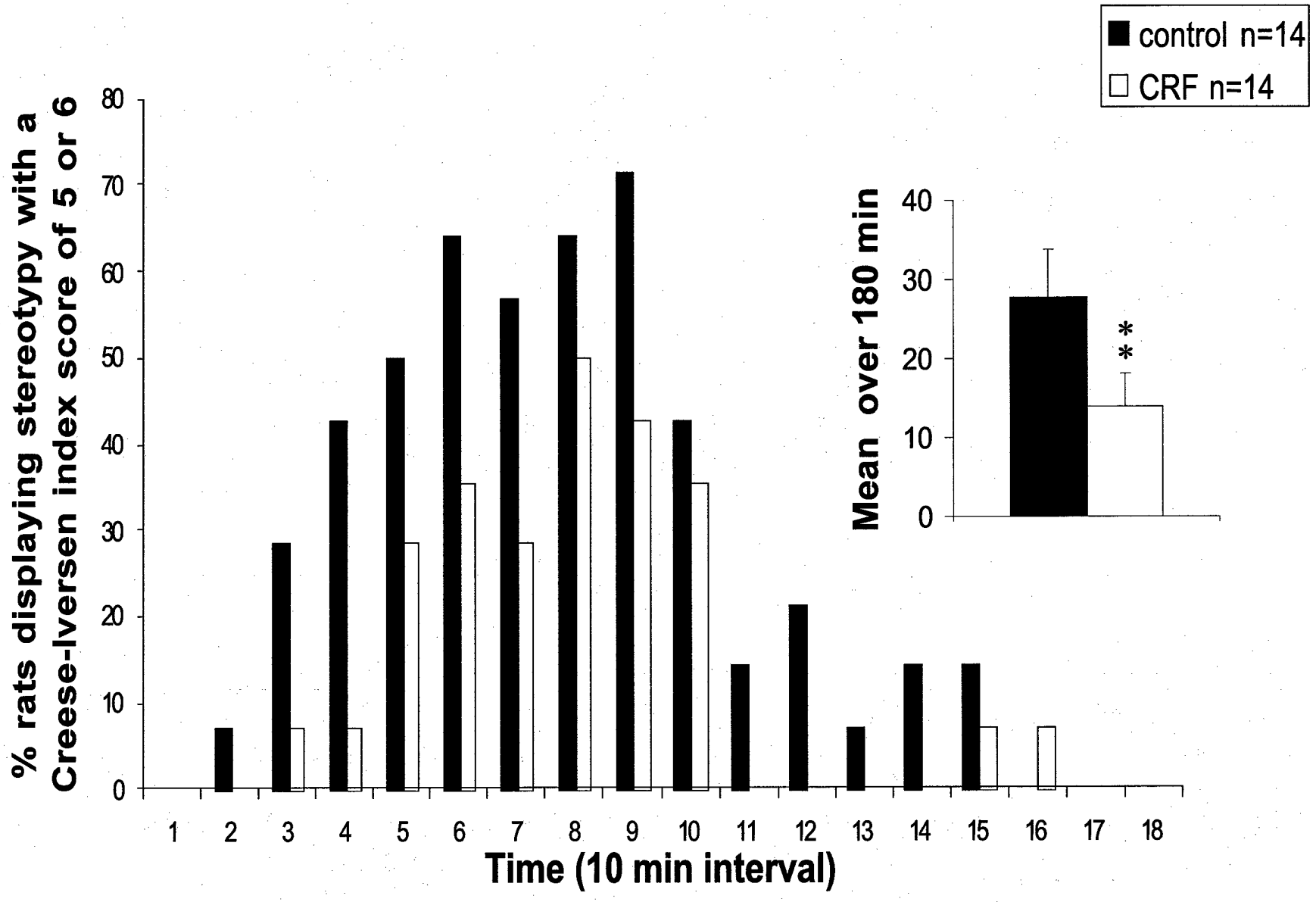
Stereotypy behavior 1 week after chronic treatment: median of the score (Creese-Iversen index) for each time point for each group

Time (min)	*10*	*20*	*30*	*40*	*50*	*60*	*70*	*80*	*90*	*100*	*110*	*120*	*130*	*140*	*150*	*160*	*170*	*180*
Control	4	4	4	4	4	4	4	4	4	4	4	3	3	3	3	3	2	2
CRF	3	4	4	4	4	4	4	4	4	4	3	3	3	3	3	3	2	2

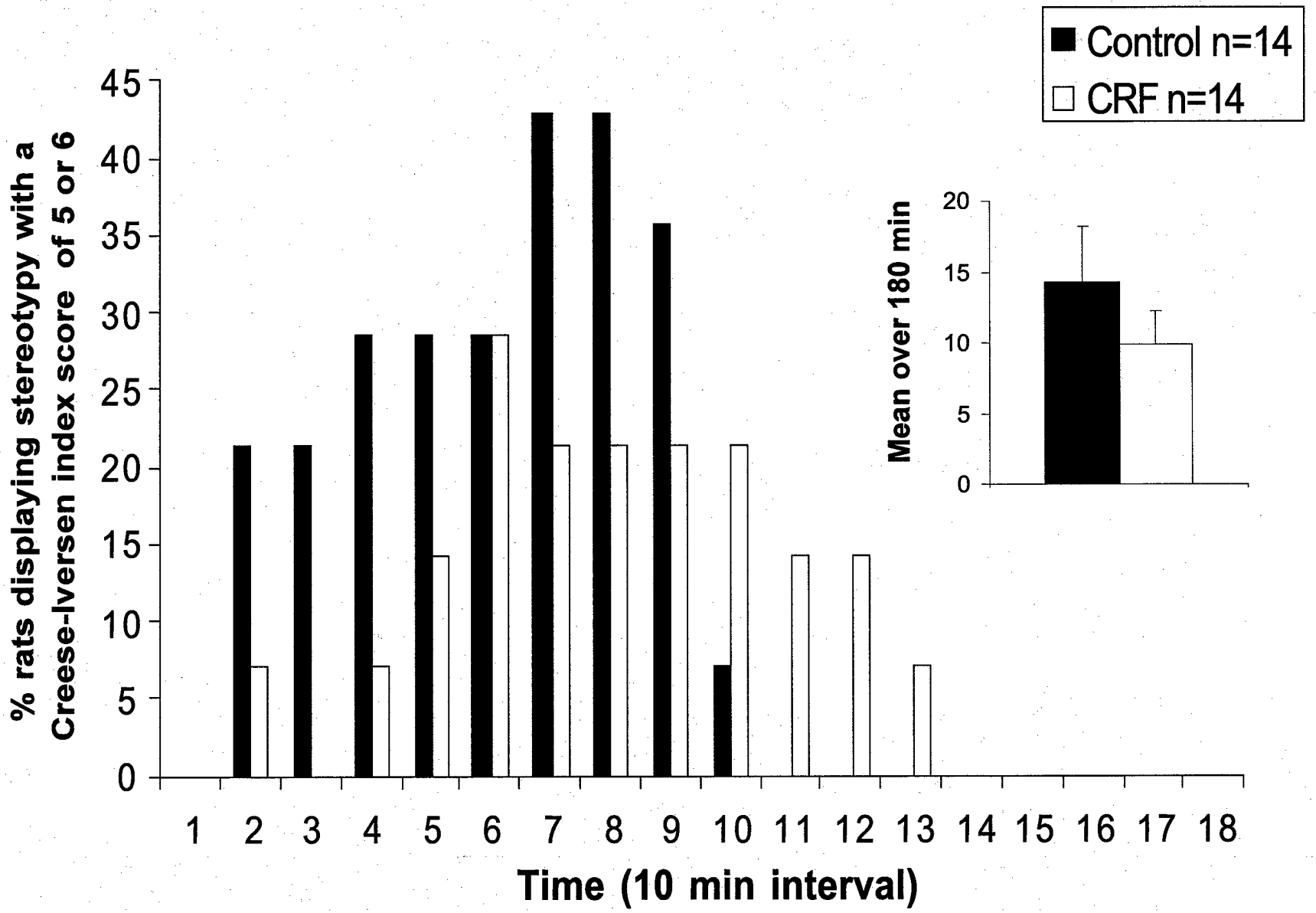
Table 3:

Stereotypy behavior 1 month after chronic treatment: median of the score (Creese-Iversen index) for each time point for each group

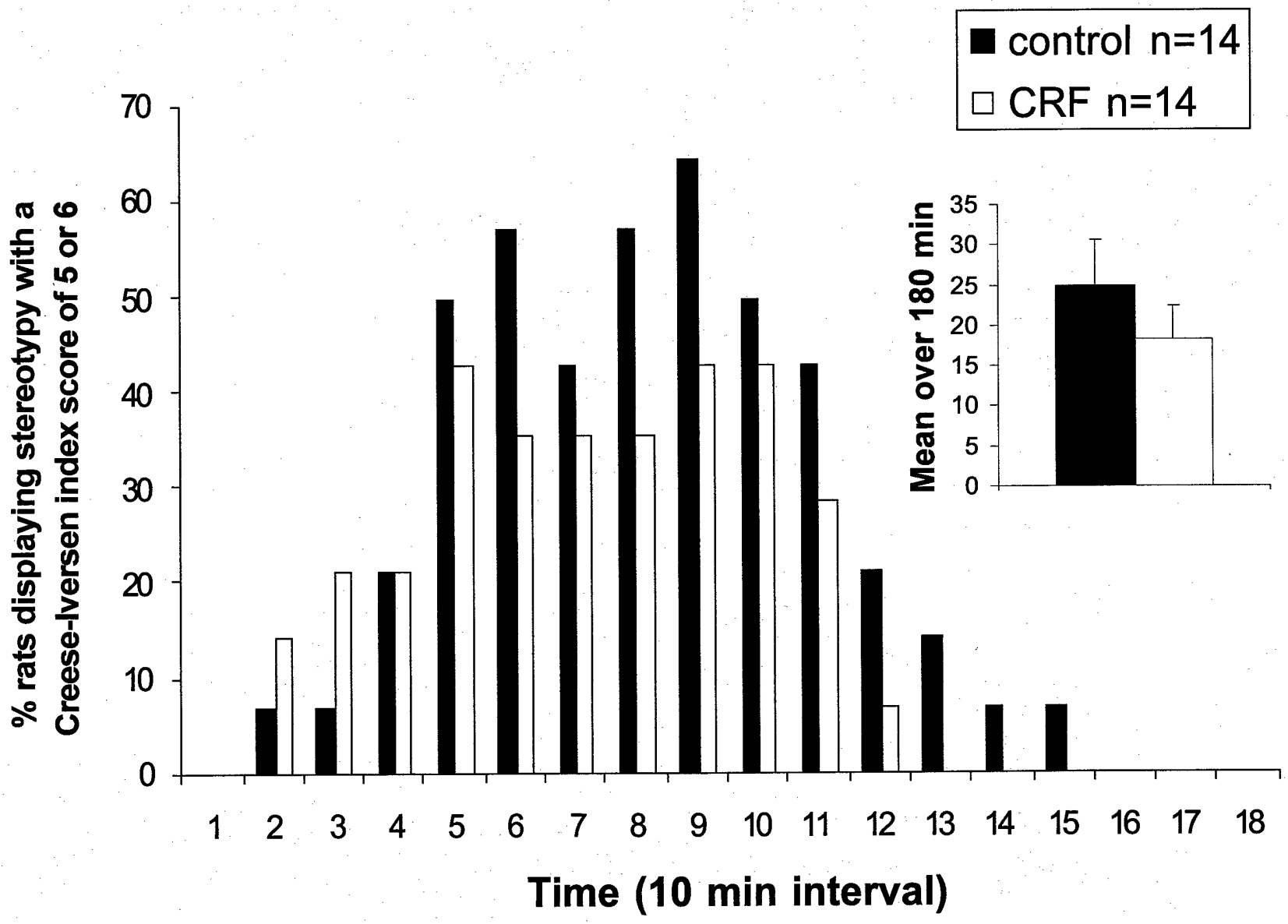
Time (min)	*10*	*20*	*30*	*40*	*50*	*60*	*70*	*80*	*90*	*100*	*110*	*120*	*130*	*140*	*150*	*160*	*170*	*180*
Control	3	4	4	4	5	5	4.5	5	5	5	4	4	3	3	3	2	2	2
CRF	3	4	4	4	4	4	4	4	4	4	4	4	3	3	2.5	2	2	2



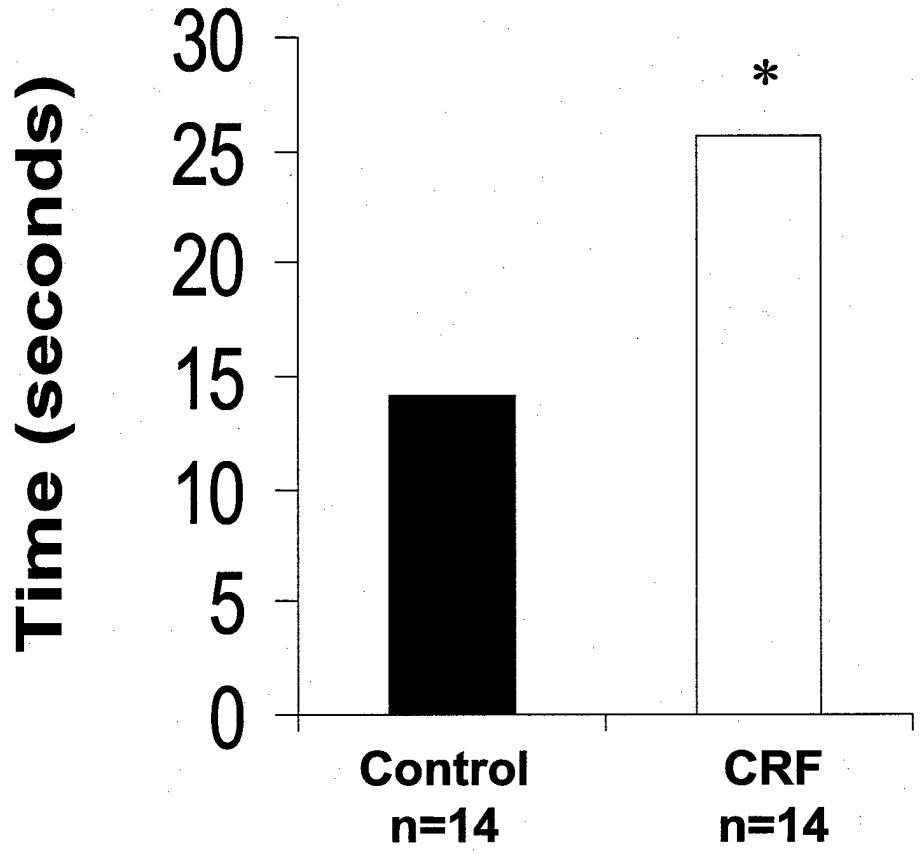
Izzo et al, fig 1



Izzo et al, fig 2



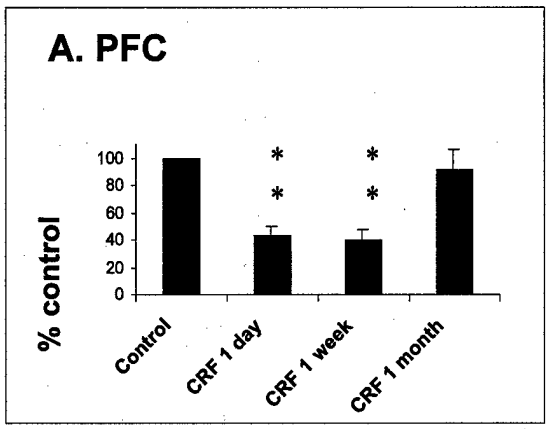
Izzo et al, fig 3

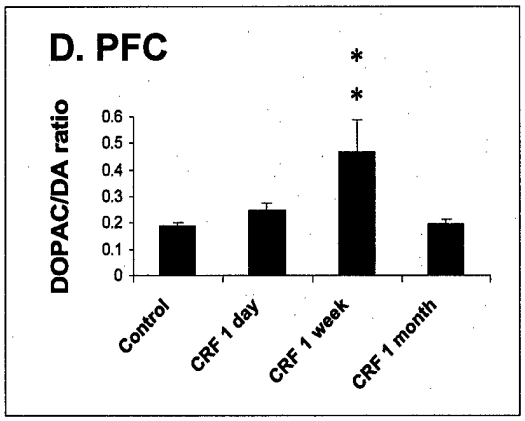


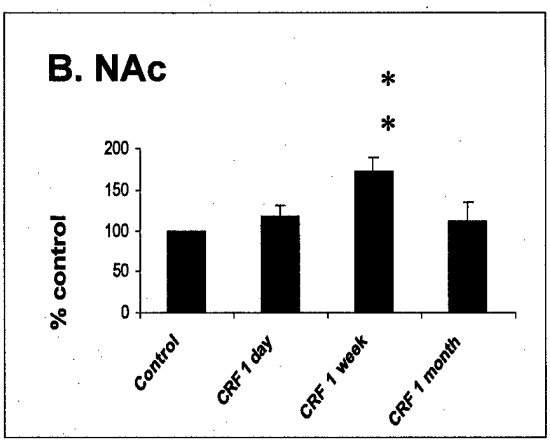
Izzo et al, fig 4

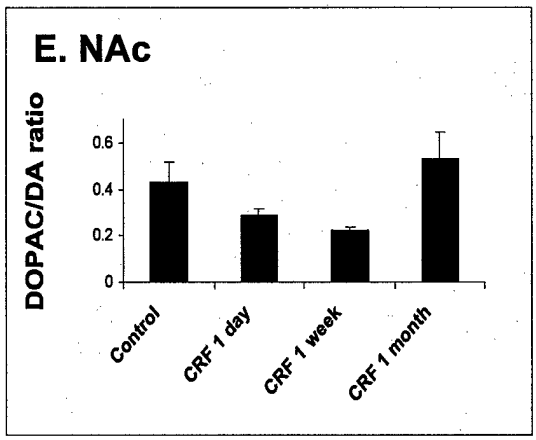
Dopamine

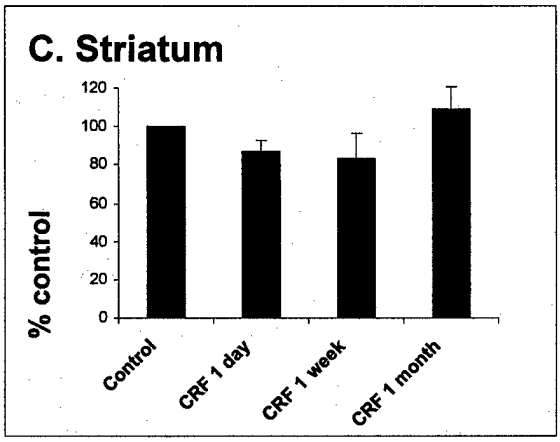
DOPAC/DA

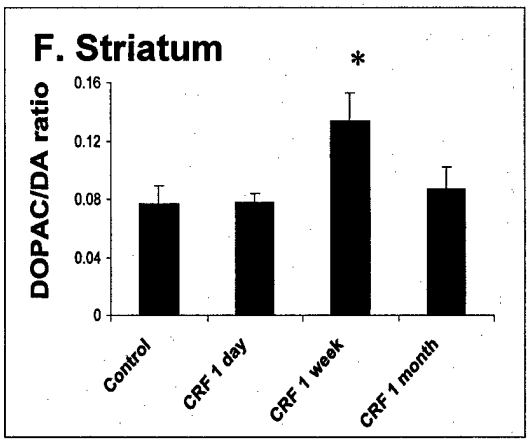




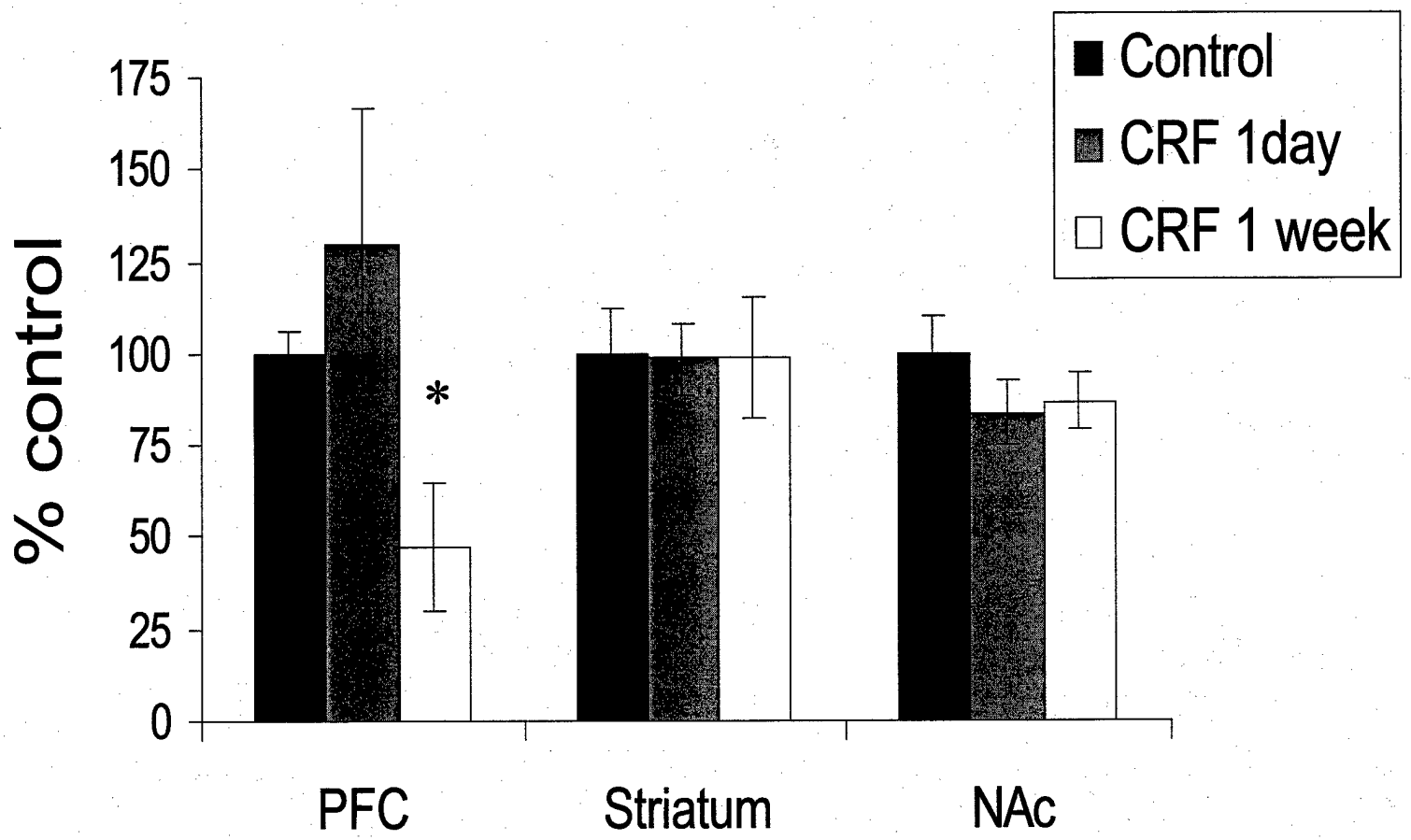








Izzo et al, fig 5



Izzo et al, fig 6